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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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BIOLOGICAL BULLETIN

INITIATION OF DEVELOPMENT IN NEREIS.

E. E. JUST.

(*From the Marine Biological Laboratory, Woods Hole, Mass., and the Physiological Laboratory, Howard University School of Medicine, Washington, D. C.*)

If any apology be needed for merely adding to the long list of eggs susceptible to agents of artificial parthenogenesis it may be suggested that initiation of development in annelids possesses some interest since annelid ova respond only with difficulty to agents that induce development.[1] We need but recall the case of *Chætopterus* (Loeb, '01*b*, Lillie '02, Allyn), of *Amphitrite* (Loeb, '01*a*, Scott), of *Podarke* (Treadwell), of the Pacific *Nereis* (Loeb, '13), of *Polynoe* (Loeb, '07, '08) and of *Nereis limbata* (Fischer, Lillie, '11, Loeb, '12*b*, '13)[2] to justify this statement. In all these eggs differentiation without cleavage is far easier to obtain than development closely simulating the normal. Among diverse agents few only will give cleavage in *Chætopterus* (Allyn, Loeb and Wasteneys). The case of *Thalassema* stands almost alone among annelids in giving development which is, according to Lefevre, to a surprising degree like the normal. Another instance among annelids of differentiation with cleavage artificially induced is worthy of note.

This report, however, on the initiation of development in *Nereis* by heat has, I think, special significance. The results here offered require an interpretation which concerns the fundamental theory of parthenogenesis and fertilization.

Experimental.

Certain preliminary experiments date from 1913. The experiments given here were performed during June, July, and

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[1] Bullot claims to have produced normal development in *Ophelia* with artificial means.

[2] Loeb's experiments with *Nereis* ('12) were apparently incomplete.

August 1914, at the Marine Biological Laboratory, Woods Hole. The majority of the experiments deal with the effect of heat on the *Nereis* egg. Under *A* these experiments are described. Under *B* are described experiments with KCl.

A. The Effect of Warming on the Initiation of Development in Nereis.

Methods.—At first all sea-water used was heated, usually not beyond 75° C., to destroy any spermatozoa possibly present, cooled, and vigorously shaken before the experiment. But this is unnecessary, as my observations showed. I have kept *Nereis* eggs in sea-water during the cool days of June for thirty-six hours without even jelly secretion. During several seasons I have never found eggs spontaneously developing in sea-water, although eggs occasionally extrude part of their jelly. Moreover, in not a single uninseminated control in ordinary sea-water was a developing egg ever found. In many experiments in addition to the uninseminated control a batch of eggs from the same animal as those warmed was inseminated. It was thus clearly proved that the eggs subjected to warming are in no wise abnormal. For fear of contamination, the needless inseminated control was discarded in the later work.

For a given experiment the following procedure was adopted: A small flask or a large test tube with a measured quantity of sea-water was placed in a large beaker of sea-water. This was warmed over an alcohol flame and the temperature kept constant by the use of thermometers in the flask and in the beaker. The eggs were generally from one female; if from several small ones, they were mixed so that the inseminated or uninseminated controls and the warmed eggs were always the same. The eggs in the initial experiments (see below) were either from females cut in the warm sea-water or they were put in the warm sea-water dry; *i. e.*, from a thoroughly dried female which was pricked to cause the escape of eggs. Eggs were also subjected to heat after washing by changing the sea-water several times during various intervals of time. By means of a capillary pipette measured quantities of eggs were transferred after exposure at varying intervals to five or to one hundred c.c. of ordinary sea-water. The experiments were performed during the morning and afternoon following the evening that the worms were captured. A few experiments were performed during the evening of capture.

The point to be emphasized is that washing in sea-water so modifies the eggs that they do not respond readily, or at all, to parthenogenetic treatment.

The Experiments.

The experiments with heat may be divided into four groups as follows:

1. The initial experiments in which the eggs were cut from the animals while in the warm sea-water.
2. The experiments with dry eggs.
3. The experiments with eggs in warm "serum."
4. The experiments with washed eggs.

1. The Initial Experiments.

In the initial experiments, worms were cut in 5, 10, 25, 50, and 100 c.c. of sea-water at 30°, 32°, 33°, 34°, 35°, 36° C., the worms removed and the eggs exposed for from five to fifteen minutes.

The following experiments selected from a number give the details:

(*a*) July 22, 1:45 P.M. A female put in 100 c.c. of sea-water at 31° C. swims actively without discharging eggs. At 1:50, the temperature is 35° C. Eggs are cut out, the worm removed. Ten samples of eggs are removed to 5 c.c. of ordinary sea-water as follows: 2.03, 2:10, 2:25, 2:35, 2:45, 2:55, 3:05, 3:15, 3:25, 3:35 P.M.

At times the temperature rose to 36° C. and once to 36.5° C. Many eggs at the time of removal from the warm sea-water exhibited membranes standing off at an unusual distance, others were darker than normal, and a few had disintegrated. Later experiments showed that these changes are due to exposure at too high temperature. Even five minutes exposure at 37° C. will bring them about. The jelly is formed in the warm water, and often at 35° C. or above it is dissolved and disappears. This may be shown by examining eggs in India ink ground up in sea-water. Many developing eggs are devoid of jelly hull, but the cortical changes are complete.

One hour and ten minutes after exposure, some eggs are in "blister" cleavages; that is, the protoplasm is irregularly budded. One hour and thirty minutes to two hours after the change to ordinary sea-water, among all gradations of cleavage-like patterns are some normal two and four-cell stages. The next day, Nos. 3 to 10 showed some real cleavages and a small per cent. of apparently normal swimming forms. Many are beaded or blistered, some are unsegmented "swimmers," and some two and four-cell swimming forms. Some eggs remain in the germinal vesicle stage.

(*b*) June 23, 11:00 A.M. A female placed in 50 c.c. of sea-water at 35.5° C. is rendered immobile but does not shed. Eggs are cut out at 11:02, the worm removed. Six samples of eggs are taken as follows: 11:18, 11:25, 11:34, 11:40, 11:45, 11:50.

Many eggs on removal from the warm sea-water show the jelly formed. The membranes after jelly formation are still a little farther from the eggs than in normal fertilization. Many eggs remain in the germinal vesicle stage with the cortex intact.

1:45 P.M. Fairly normal cleavages in Nos. 1 to 4.

June 24, 8:30 A.M. Swimming forms are found in the dishes. By far the best are those in Nos. 2 and 3.

The optimum time of exposure, therefore, lies between twenty-three and thirty-two minutes. Later experiments showed that the optimum exposure at 35° C. is at or near twenty-five minutes.

(*c*) June 24, several experiments were run at various temperatures. Those at 35° C. confirmed the findings of the previous ones. Temperatures ranging from 30° C. to 31° C. give no results; regardless of the length of exposure the eggs remain in the germinal vesicle stage.

The following experiment of June 24, at 33° C. is typical of a number of repetitions at this temperature:

(*d*) June 24, 11:15 A.M. A female placed in 25 c.c. of sea-water at 33° C. swims actively without discharging eggs. Eggs are cut out at 11:15 and samples taken at five-minute intervals up to 11:50. The temperature is practically constant. The samples taken are masses of eggs with the cortex wholly or (in earlier ones) partially broken down. The cytoplasm is normal in color and the membranes normal.

1:35 P.M., many eggs are in cleavage.

4:00 P.M., many eggs are in late cleavage.

June 25, 9:00 A.M. The dishes show a good per cent. of very fine "swimmers." The cleavage seems almost normal.

Thirty-five minutes' exposure gives by far the highest percentage of swimming forms. As in all the experiments of this group, some eggs remain in the germinal vesicle stage with cortex intact.

If eggs be warmed in "egg-water" (sea-water charged by eggs that have remained in it for several hours) the results are no different.

To sum up, we find that eggs of *Nereis* cut out in warm sea-water and exposed to temperatures ranging from 33° to 35° C. develop with cleavage which is closely similar to the normal. Some eggs remain in the germinal vesicle stage. For the best percentage of swimming forms the optimum exposure at 35° is twenty-five minutes; at 33°, is thirty-five minutes.

2. Experiments with Dry Eggs.

Many of the experiments with dry eggs were run with the washed egg series. In the majority of cases eggs from one female thoroughly dried on clean filter paper were received in a dry watch glass.[1] These eggs were divided into two lots; one lot warmed in sea-water at the given temperature and the other washed by changing the sea-water several times, allowed to settle, and after draining placed in the warm sea-water.

A large number of experiments was made with dry eggs, in the attempt to determine the quantitative relations early found to control the number of eggs developing. Thus, with smaller quantities of warm sea-water every single egg quickly forms jelly and at least ninety-eight per cent. cleave, but with larger quantities of warm sea-water the percentages are lower.

As Miss Allyn found for *Chœtopterus* cleavage appears to interfere with the further development. I have never been able to get more than twenty per cent. of these eggs to reach the swimming stage. If one could determine definitely the quantitative relations this percentage might be increased. From the observations it appears that the optimum amount of warm water used varies; it depends upon the bulk of the eggs. While best results are got with small quantities of water, it is possible to use too little—three c.c. for instance, for the eggs for a large female. Jelly formation and cleavage are induced but swimming forms are less numerous than in the case of ten c.c. for about the same bulk of eggs.

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[1] With dry eggs one must be careful for the mere drying will initiate changes as I have found. Eggs left on filter paper for from five to twenty minutes form jelly, a small per cent. cleave and a few swim.

The following are typical experiments of this group:

(*a*) July 16, 10:30 A.M. Eggs from a dry female in a dry watch glass are divided into two lots; one lot washed, the other transferred to 5 cc. of sea-water at 34° C. Samples out as follows: at 10:20, 10:50, and at five-minute intervals thereafter to 11:20.

2:00 P.M. Uninseminated control, no change. *Every single warmed egg had formed jelly: all have formed polar bodies.* At least half of these are in cleavage stages.

July 17. All dry eggs in some stage of cleavage, many of which are normal; some swimming forms in many of the dishes even after forty minutes' exposure.

(*b*) July 17, 9:58 A.M. Eggs from a dry female divided into two lots. Lot *A* in 5 c.c. of sea-water; Lot *B* in 20 c.c. of sea-water. Both exposed to 33° C. 10:01 jelly formation. Eight samples taken as follows: 10:05, 10:11, 10:16, 10:22, 10:27, 10:33, 10:38, and 10:43.

Lot *A* gave at least 95 per cent. of cleavage and a percentage of swimming forms in all dishes beginning with No. 3 (the 18-minute exposure). Lot *B* gave 75 per cent. of cleavage and best swimmers for 24, 29, and 35-minute exposures.

(*c*) Other experiments showed that the highest per cent. (100 per cent.) of jelly formation and of cleavage (98 to 99 per cent.) is in the smaller quantities of sea-water—5, 6, and 10 c.c. —whatever the temperature; a few swim. With larger quantities of sea-water at the various temperatures more eggs remain in the germinal vesicle stage. The lower exposures give most normal-looking swimming forms—trochophores scarcely to be distinguished from the normal either while living or in sectioned material. The higher exposures give more abnormal swimming forms.

For comparisons I have selected the following tables from my notes to show the percentages of cleavage and of "swimmers" obtained with eggs from worms cut in warm sea-water and with dry eggs. It is apparent at once that while there is no appreciable difference in the percentages of swimming forms after warming either the "cut out" or the dry eggs, there is a marked difference in the percentages of cleaving eggs. This is the case in all the experiments.

July 12. Two females cut up at 9:55 A.M. in separate flasks of sea-water at 35° C. gave the following results:

Female No. 1.

	Sample Taken	Cleavage.	Swimming Forms.
No. 1	10:06	65%	1.5%
" 2	10:13	50	4
" 3	10:15	55	5
" 4	10:20	67	5
" 5	10:25	60	9
" 6	10:30	72	3
" 7	10:35	47	7
" 8	10:40	68	4—abnormal
" 9	10:45	62	very abnormal
" 10	10:50	33	very abnormal

Female No. 2.

	Sample Taken	Cleavage.	Swimming Forms.
No. 1	10:07	66%	3%
" 2	10:14	42	8
" 3	10:16	33	12
" 4	10:21	47	14
" 5	10:26	44	3
" 6	10:31	74	10
" 7	10:36	24	2—abnormal
" 8	10:41	81	5— "
" 9	10:46	32	very abnormal
" 10	10:51	17	very abnormal

July 20. Dry eggs in 30 cc. of warm water at 35° C. gave the following results:

Exposure.	Cleavage.	Swimming Forms.
18 minutes	90%	1%
23 "	"	15%
30 "	"	10%
35 "	"	7%

With dry eggs one may obtain 100 per cent. cleavage; with the eggs cut from worms in warm water one never gets more than 81 per cent. the average being very much lower as the figures given above show. With both kinds of eggs 20 per cent. swimming forms is the maximum, the optimum exposure for the various temperatures used being the same.

Experiments show that the use of warm "egg-water" does not improve the results.

3. The Experiments with Serum Eggs.

The effect of warming *Nereis* eggs in the body fluids was studied with difficulty mainly because of the scarcity of body fluid in *Nereis*. As Lillie has pointed out this worm is little more than a bag of eggs. The amount of blood present is negligible and unavailable for warming experiments. I therefore adopted the method used by Lillie—that of cutting up spent females. In his study this juice gave results comparable to the perivisceral fluids in *Arbacia*. For an experiment I minced as many spent females as I could get, using a small quantity of sea-water; the juice thus obtained is designated as "serum." While I think that my experiments with this serum are conclusive I wish to point out that *Nereis* is not the most favorable form with which to establish the fact of serum inhibition—certainly this is true for the method I used. It may be stated at the outset that as Lillie found for both *Nereis* and *Arbacia* I have found repeatedly that the "serum" of *Nereis* quite definitely inhibits fertilization. Furthermore, just as definitely does the serum inhibit initiation of development with warming. I cite experiments to give the details:

(*a*) July 15, 10:55. Eggs from one fine large female previously dried are divided into four lots. Eleven spent females are finely minced to procure twenty drops of "serum." Ten drops of the "serum" is added to each of two dishes containing 3 c.c. of sea-water; eggs added to both. One lot is warmed at 34.5° C.—Lot *A*; Lot *B* inseminated. Samples of *A* are taken at five-minute intervals up to 11:35. 2:00 P.M., 1 per cent. of cleavage in both lots. Next day no swimming forms in either. Eggs from the same female, Lot *C*, warmed in sea-water and Lot *D*, inseminated, develop.

During the afternoon of July 15 this experiment was repeated with the same results.

(*b*) July 22. Eggs warmed at 34° C. in serum plus sea-water (serum from the bodies of seven spent females cut up in two c.c. of sea-water): 10 drops plus three c.c. sea-water, 10 drops plus five c.c. sea-water and 10 drops plus ten c.c. sea-water.

Eggs exposed for twenty-five minutes. Less than one per cent. developed in any dish.

During August these results with serum eggs were verified. The highest per cent. of swimming forms obtained was one per cent.; this was with a very dilute serum. Not only do the eggs fail to cleave but fail in the great number of cases even to form jelly. In some cases the development of eggs inseminated in serum was farther advanced than the serum warmed eggs. Since in the case of the initial experiments the worms were cut up in sea-water, it may be that failure of a percentage of eggs to cleave is due to the inhibition of the escaping blood and tissue juice. With the dry eggs cut quickly on the dry watch glass this escaping juice cannot so easily contaminate the eggs.

4. The Experiments with Washed Eggs.

In *Platynereis* sea-water definitely destroys the fertilizing power of the egg. Even minute quantities of sea-water will render the egg incapable of cleavage although the spermatozoa may penetrate. Moreover, if the eggs of one female remain in a small quantity of sea-water, 5 c.c., for instance, for *thirty seconds* their fertilizing power is lost. And yet in nature, inseminated eggs begin to be laid in many cases *five or six seconds* after copulation (see Just, '14). In *Nereis*, therefore, it was thought that washings in sea-water by frequent changes through several hours might act as the sea-water does in such a surprisingly short time on *Platynereis* eggs.

During the June *Nereis* run, then, as many experiments as possible were conducted to determine the "fertilizable" period by inseminating at intervals eggs that had remained in sea-water with and without frequent washings. Lillie has shown for *Arbacia* eggs that the capacity for being fertilized decreased with the decreased secretion of fertilizin. He finds for *Nereis* also very much the same relationship.

Without going into details, it may be said at the outset that the egg of *Nereis* gradually loses its power of being fertilized and eventually reaches the condition of the *Platynereis* egg where insemination induces maturation only. I cite a single experiment.

June 28, 9:10 P.M. Dishes of eggs Nos. 1, 2, and 3 were set aside. The next day at 2:10 P.M. each dish of eggs was drained and divided into two lots—*A* and *B*. Lot *A* in each case was inseminated in the water which had stood over the eggs for seventeen hours. Lot *B* of each dish was inseminated in fresh sea-water. No eggs in either lot of No. 1 developed beyond maturtion. In Lots *A* and *B* of Nos. 2 and 3, .1 per cent. or less went as far as the two-cell stage. Some eggs in all the dishes were in the germinal vesicle stage. No trochophores were found.

Eggs were frequently tied in bags of filter paper and placed in a beaker under running water for twelve hours. In other cases they were washed by changing the water at odd times during the day. It was found that eggs differ greatly with respect to the time that they must remain in sea-water before they lose their fertilizing power, but it may be clearly proved that washing or staling of *Nereis* eggs renders them incapable of being fertilized. This stage may be reached after three hours in seawater (cf. Just, '12).

This varying susceptibility proved very disappointing because I had suspected, not, of course, the degree of susceptibility present in *Platynereis*, but perhaps such as could be expressed more definitely.

Because of these results with washed and stale egg insemination, when the warm sea-water experiments were continued during the July *Nereis* "run" I was certainly unprepared for the results obtained. The following experiments are typical of a large number performed almost daily during the July and August "runs":

(*a*) July 11, 9:50 A.M. Eggs from one female divided into two lots; one lot put in sea-water. This lot transferred from the sea-water to warmed sea-water (35° C.). The eggs form jelly in the warm sea-water and make a mass which has to be shaken to obtain samples. 10:55, many have formed jelly and maturated, but most retain jelly with germinal vesicle intact. Some of these eggs again subjected to heat; no results. July 12. Very few, 1 in 1000, swimming.

(*b*) July 15, 3:20 P.M. Eggs cut out and washed, put in 6 c.c. of sea-water warmed at 35° C. Samples taken at five-minute intervals for forty minutes. Next day: Majority are in germinal vesicle stage, at least seventy-five per cent. Less than one per cent. swimming.

(*c*) July 16, 9:35 A.M. Eggs washed ten times evening before and five times during this morning. Two series: *A* inseminated, *B* in warmed sea-water at 3:50 samples taken (ten in all) at five-minute intervals. Uninseminated control.

July 17, 1:30 P.M. No development in uninseminated control (few have cytolyzed). Inseminated eggs show that few have formed jelly (ten to fifteen per cent.). One per cent. have cleaved and some of these swim. Of the warmed eggs at least ninety-five per cent. are in the germinal vesicle stage with cortex intact. Less than one per cent. have formed polar bodies.

(*d*) July 16, 10:30 A.M. Eggs from a dried female divided in two lots; one lot washed in 100 c.c. of sea-water by changing the water four times. 10:40 A.M. In warmed sea-water, 34° C. Samples out at five-minute intervals for sixty minutes.

2:00 P.M. At least ninety per cent. in the germinal vesicle stage, small per cent. form jelly and divide. Next day, none swim.

I was tempted to discredit my June experiments after the first of these findings. I could only convince myself after running series after series of washed and dry eggs along with eggs cut out directly into warmed sea-water. Most workers in inseminating eggs obtain the sexual products in separate dishes, and add sperm. Such procedure succeeds admirably with *Nereis* giving one hundred per cent. of cleavage. But if eggs be cut out of *Nereis* in sea-water, divided in two lots, and washed once or twice, one lot being inseminated and the other warmed we get the surprising result that while every single inseminated egg develops, few of the warmed go beyond maturation. If the water over the eggs be changed a few times in ten minutes, ninety per cent. warmed in sea-water fail even to maturate.

This must mean that the egg of *Nereis* is so susceptible to sea-water that warming fails after washing although fertilization is still possible. If fertilization be impossible (as in stale eggs) warming also produces no effect.

Washed or stale eggs warmed in sea-water charged by eggs that have remained in it for some time do not fare any better than those subjected to warmed sea-water; as in the first and second series of experiments this "egg water" makes no difference in the results.

I think that these facts are incontrovertible. Washing or even residence in sea-water for a short time interferes seriously with the effect of heat in initiating development.

Study of insemination of dry and washed eggs was made. Apparently there is a difference here of response to the spermatozoon. The dry egg is more irritable, jelly formation being extremely rapid. This is true of dry eggs inseminated in small quantities of sea-water. This behavior recalls that of *Platynereis.*

These results, moreover, might suggest that our methods are much too crude in the study of these extremely sensitive cells—the egg and the spermatozoon.

Summing up we may say concerning the effects of warming on the eggs of *Nereis*: (1) *That while eggs cut out of worms in the warm sea-water form jelly and divide in large numbers, a small per cent. swimming, some remain in the germinal vesicle stage.* (2) *That at least ninety-eight per cent. of the dry eggs form jelly almost all of which cleave: twenty per cent. become trochophores closely resembling the normal.* (3) *That eggs in "serum" fail to develop except in very small numbers.* (4) *That washed eggs even after but two or three washings develop if at all in small numbers.*

B. EFFECT OF KCl IN THE INITIATION OF DEVELOPMENT.

According to Fischer the eggs of *Nereis* after treatment with KCl will go through cleavage and produce trochophores. Lillie ('11), however, could not get the eggs after KCl treatment to go beyond maturation. During three seasons this had been my experience. This summer I studied the effect of KCl on washed and unwashed eggs.

If the eggs be washed two or three times before exposing to the action of KCl every egg maturates but never more than one in a thousand swims. If the eggs be allowed to remain in sea-water from two to twelve hours with frequent changes of sea water the results are about the same. Dry eggs subjected to KCl treatment maturate, cleave once or twice, and produce, in one experiment at least, seven per cent. of swimming forms made up of unsegmented two and four-cell "swimmers."

The Experiments.

5, 10, 15, 20 and 25 per cent. 2.5M KCl were used. It was found that 15 per cent. 2.5M KCl in sea-water gave the best results. Typical experiments follow:

(*a*) August 12, 10:54 A.M. Lot *A*: Eggs from two females cut in 80 c.c. of 20 per cent. 2.5M KCl at 10:54. Lot *B*: Eggs from one dry female put in 3 c.c. of 20 per cent. 2.5M KCl. At 11:00 jelly formation in both. Samples of eggs taken from *A* and *B* as follows:

No. 1	11:00
No. 2	11:10
No. 3	11:17
No. 4	11:25
No. 5	11:33
No. 6	11:40
No. 7	11:50
No. 8	12:00

August 13, 12:00 M. Dry eggs of August 12 (Lots *A* and *B*). All maturated; some cleavage-like processes and some swimming forms after twenty minutes' exposure or more. Highest percentage (five) of swimmers after fifty minutes' exposure. These are unsegmented, two and four-cell swimmers.

(*b*) August 12, 12:05 P.M. Water changed three times on eggs during three hours and then placed in 20 c.c. of 20 per cent. 2.5 M KCl in sea-water. Samples taken at five-minute intervals up to 1:00 P.M.

August 13, 1:00 P.M. Washed eggs of August 12, all maturated; 1 in 1,000 swim.

Experiments during August 13 and 14 with fifteen per cent. 2.5 M KCl gave about the same results.

(*c*) August 15, 9:30 A.M. Two females quickly cut in 10 c.c.

of 15 per cent. 2.5 M KCl and removed. Samples taken as follows:

No. 1	10:35
" 2	10:50
" 3	11:05
" 4	11:20
" 5	11:40
" 6	12:00
" 7	1:25

These gave the next day swimming forms as follows:

No. 1	4 per cent
" 2	7 " "
" 3	4 " "
" 4	2 " "
" 5	2 " "
" 6	2 " "
" 7	3 " "

(*d*) On August 17, eggs were washed by changing the water six times in five hours then subjected to 15 per cent. 2.5 M KCl in sea-water, samples being taken at sixty minutes and thereafter at five-minute intervals. One tenth per cent. (.1%) was the best result after sixty minutes in the KCl sea-water.

(*e*) *Combination of KCl with Heat.*[1]—An experiment of last summer was repeated except that dry eggs were used and the minimum exposure, five minutes, of the series the only one tried. The protocol follows:

August 17, 1:25 P.M. Dry eggs are put in 10 c.c. of 15 per cent. 2.5 M KCl in sea-water for five minutes; jelly formation almost at once. Eggs are then placed in 50 c.c. of sea-water at 35° C. Four lots removed to 100 c.c. of sea-water as follows:

No. 1	1:55
" 2	2:00
" 3	2:05
" 4	2:10

The next day, at 10:30 A.M., the percentages of swimming forms, largely unsegmented found were as follows:

No. 1	(25 minutes' exposure to heat)	3 per cent.
" 2	(30 " " " ")	7 " "
" 3	(35 " " " ")	27 " "
" 4	(40 " " " ")	25 " "

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[1] It will be recalled that Allyn used a combination treatment of KCl and heat on the egg of *Chætopterus* with rather different results from those mentioned here with *Nereis*. Her method however, was different.

It appears, therefore, that with KCl, and KCl and heat, washed and unwashed eggs alike will maturate, but that the dry eggs alone respond with cleavage or the production of swimmers.

Discussion.

In the egg of *Nereis* Lillie discovered a substance, *fertilizin*, which has the property of agglutinating *Nereis* sperm. This substance may be detected in the water in which the eggs have remained for a short time. If, however, the eggs be washed by changing the water two or three times the fertilizin is no longer secreted in detectable quantities, *i. e.*, there is not enough to agglutinate the sperm. Such eggs are none the less fertilizable by sperm, giving off at the time of insemination more fertilizin, all of which is then utilized or completely thrown off during the cortical changes. It therefore follows that at the time of shedding the egg is laden with free fertilizin ready for secretion. This conclusion is supported by additional facts. In the first place I have pointed out above that the dry egg or egg in small quantities of sea-water is hyper-irritable—that is, if jelly formation may be taken as index. If one inseminates the eggs of *Nereis* dry or in small quantities of sea-water the jelly formation is extremely rapid. Jelly formation is correspondingly slow in washed and stale eggs. The breeding behavior noted night after night for several seasons is significant: freshly shed eggs at the surface of the sea excite numbers of males to shed their sperm around the shedding or recently spent female. Lillie's experiments (Lillie and Just) on this sperm shedding reflex, moreover, prove that the egg loses fertilizin once in the sea-water. The "dry" and "washed" eggs of my experiments, then, are physiologically different: the dry egg has all its available fertilizin content, the washed egg has secreted part of this substance.

Lillie has shown that the eggs of *Nereis* will not fertilize in the tissue juices of the animal; my experiments show also that the body juice of spent females inhibits fertilization. Unlike the washed egg, the "serum" eggs possess fertilizin but its action is inhibited.

But it is on the basis of experiments on *Arbacia* that Lillie has developed the fertilizin theory as an explanation of the mechanism of fertilization. Without going into details it may be said that in *Arbacia* it is found that the egg secretes a substance, fertilizin, whose presence is capable of quantitative determination and which is necessary for fertilization since first, eggs washed free of it are no longer capable for fertilization; second, fertilized eggs no longer secrete it; and third, eggs after membrane formation with butyric acid are not capable of fertilization and do not give off the substance. The perivisceral fluid of *Arbacia*, moreover, produces an inhibiting effect on fertilization preventing the action of fertilizin on the egg.

My results with warming *Nereis* eggs parallel to a striking degree these facts brought out in the studies of fertilization in *Nereis* and *Arbacia* (Lillie, '12, '13*a*, '13*b*, '14). Eggs washed free of the bulk of fertilizin will not develop however long the warming treatment lasts; serum inhibits the artificial initiation of developmental processes; only the dry eggs with their full content of fertilizin when suddenly shocked with elevation of temperature respond with jelly formation and cleavage. It would seem, therefore, as I have suggested for *Platynereis*, that fertilizin is just as essential for artificial initiation as for normal fertilization. The difference seems to be that for artificial initiation more fertilizin is required. Further attempts at Woods Hole this summer to induce artificial parthenogenesis in *Platynereis* strengthen this belief; a percentage of *Platynereis* eggs will fertilize in small quantities of sea-water; the same bulk of eggs in the same amount of water fail to respond when subjected to warming.

If, therefore, as Loeb ('12*a*) says, "fertilization is primarily and essentially artificial parthenogenesis"; or if "a theory of fertilization must also be a theory of parthenogenesis at least for the phenomena common to both"; and if "similarly a theory of fertilization must be consistent with the facts of parthenogenesis" as Lillie ('14) suggests; these experiments, we are forced to conclude, make another link in the chain of evidence which supports the theory that fertilization is essentially a process of the egg. The spermatozoon initiates the development of the egg, as does warming, through the activation and the binding of the fertilizin.

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SPERM AGGLUTINATION AND FERTILIZATION.

FRANK R. LILLIE.

In a recent paper on "Cluster Formation of Spermatozoa Caused by Specific Substances From Eggs" Loeb ('14) has presented a criticism of my theory of fertilization (Lillie '13*b* and '14), based on observation of the California sea-urchin *Strongylocentrotus purpuratus*. My own observations were made on *Arbacia punctulata* of Massachusetts, and it would appear that part at least of Loeb's criticism was due to certain differences in the two forms, for he has now stated (Loeb, 1914*b*, p. 318, footnote) that the "cluster formation" of the spermatozoa may find its explanation "on the assumption of an agglutination at least in the case of *Arbacia*," as I maintained; it is therefore not a "tropistic reaction" as he thought probable from his observations in California. This was one of the chief differences of opinion. A second one was in regard to the source and significance of the substance in the fluid of egg suspensions that caused such agglutination; Loeb maintained that it was merely the dissolved chorion (*i. e.*, jelly layer) of the egg, and that after this was removed the eggs no longer produced the agglutinating substance, and yet were capable of fertilization; whereas my contention was that the agglutinating substance was a secretion of the egg soaked up by the jelly, as by a sponge; that the eggs produced it for a certain length of time after the removal of the jelly, and lost their power of fertilization after they ceased to produce it.

These criticisms cut at the foundation of my theory. Inasmuch as the correction of the tropistic interpretation of agglutination is given only in a footnote to another paper, and no correction of the source of the agglutinating substance has yet appeared, it is incumbent on me to consider the criticisms carefully; at the same time I wish to take the opportunity to explain certain points that appear to be open to misinterpretation, and to record some new observations.

1. "CLUSTER-FORMATION" VERSUS AGGLUTINATION.

The phenomena exhibited by sperm suspensions of *Arbacia* with which we have to deal are of four distinct types, which it is essential to distinguish sharply: (1) activation; (2) aggregation; (3) agglutination, (4) mass-coagulation.[1] (1) That the activity of spermatozoa is affected by substances in the sea-water requires no argument. The subject is discussed in study V (Lillie, 1913*a*, pp. 519–532). (2) Aggregation of spermatozoa may be brought about by tropistic reactions. In my paper on the "Behavior of Spermatozoa," I devoted a great deal of attention to such aggregation phenomena and the distinction from phenomena of agglutination (1913, pp. 532–548 and pp. 551–552). Among other things I pointed out that aggregation as a tropistic phenomenon implies a gradient,[2] and that the spermatozoa never adhere, however crowded they may be; there is no observable physical change of the spermatozoa and the slightest agitation suffices to disperse them again. Such tropistic phenomena may be exhibited in response to CO_2 and other acids (*Nereis*), or certain constituents of egg secretions, to mention only chemotaxis.

(3) Agglutination of spermatozoa on the other hand requires no gradient, and the spermatozoa adhere physically to such an extent that the agglutinated masses may be preserved intact in killing fluids; its degree is a function of the concentration of the agglutinating medium, and is also different in different species. Agglutination is non-toxic, not limiting the life of the spermatozoa; it is reversible, its duration depending on the concentration of the agglutinating medium; it cannot be repeated if the reaction is complete, at least within the time limits of my experiments, even though the spermatozoa remain motile;[3] finally motility of the spermatozoa is a prerequisite to a decided reac-

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[1] The reaction here referred to is a lethal phenomenon. It possibly involves cytolysis with subsequent adhesion of the spermatozoa.

[2] It is important to notice that the spermatozoa of suspensions may produce gradients through their own activities. Thus I pointed out that autogenous aggregation reactions in sperm suspensions of *Nereis* arise from the positive chemotaxis of the spermatozoa to their excreted CO_2, giving rise to very striking phenomena (Lillie, 1913*a*, pp. 519–521 and pp. 538–540). It is conceivable that such a tropistic phenomena is involved as a part factor in the agglutination phenomena under discussion.

[3] Glaser (1914) also comes to this conclusion.

tion; evidently because the physical change on which the reaction depends is not sufficient to cause adhesion except when the spermatozoa positively collide.[1] These six criteria definitely define the phenomenon.

Agglutination is positively distinct from aggregation. It is an entirely different biological phenomenon. The two may, however, be exhibited simultaneously, as when a drop of egg secretion of *Arbacia* is injected into a sperm suspension of the species. In such a case the spermatozoa exhibit positive chemotaxis to one constituent of the egg secretion, and are agglutinated by another (the fertilizin). The separateness of these two substances was maintained in my first publication on the subject and demonstrated by repeated experiments (see Lillie, 1913*a*, p. 549, and 1914, pp. 545–546).

(4) The phenomenon of mass-coagulation is, on the other hand, a lethal irreversible phenomenon. It may be exhibited in response to various agents, such as KOH, NaOH, salts of lanthanum and cerium,[2] etc., and in some cases the secretions of the eggs of other species or their blood. Hitherto I have not adequately defined this phenomenon as distinct from the agglutination phenomena, though in my last study (1914), I noted the distinction (p. 541). The phenomenon is essentially lethal, but not all destructive agents exhibit it; thus acids, so far as I have observed, destroy the spermatozoa without causing mass coagulation. The phenomenon is irreversible, and this suffices to distinguish it from true agglutination, even if no other criterion were available. However, it exhibits quite a different aspect from agglutination; in the latter the sperm masses tend to take on a spherical form; if originally elongated they contract into balls or break up into smaller masses which become spherical, thus offering considerable resemblance to a phenomenon of surface tension, as Loeb notes. The peripheral spermatozoa are in violent movement until the time of reversal. In the mass-coagulation reaction, on the other hand, there is no such surface tension effect, strands anastomose

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[1] Loeb argues that the necessity of movement on the part of the spermatozoa for the appearance of this phenomenon removes it from the category of true agglutination; but this seems to me to be a purely arbitrary criterion.

[2] My attention was called to the action of the salts of these metals by a letter from James Gray of Cambridge University.

with other strands and form a net-work and the movements of the spermatozoa soon cease.

The substances of egg secretions, which I have hitherto called hetero-agglutinins, belong to this category, in some cases at any rate. Though I will not assert that there is no such substance as a hetero-agglutinin in the real sense of agglutination, yet the substance in *Arbacia* blood, or egg secretions, the effects of which on *Nereis* sperm I have previously studied, should be regarded as a toxic rather than an agglutinating substance, having the mass coagulant action. As I stated in my last paper, p. 541, it produces a permanent coagulum in *Nereis* sperm suspensions; "in this respect the action differs from the iso-agglutination, which is without toxic effects."

We must keep firmly in mind the distinctions between aggregation (tropisms) agglutination, and mass-coagulation. Agglutination, with which we are particularly concerned, is distinguished from aggregation by the facts that it occurs in the absence of a gradient, it involves physical adhesion, and cannot be repeated if the reaction is once complete; it also is characterized by a high degree of specificity.[1] From mass coagulation it is distinguished by the facts (1) that it is non-toxic, (2) reversible, (3) dependent on motility of the spermatozoa. Agglutination occurs so far as I have observed with certainty only in response to egg-secretions of the same species.

For description of the phenomena of agglutination of sperm by egg-extractives of the same species, I must refer to my previous paper (Lillie, 1913*a*); the phenomenon in *Arbacia* is a true agglutination in the sense defined, not a tropistic reaction, nor yet a mass coagulation. Loeb has admitted this for *Arbacia*, and I would therefore venture to suggest the probability that the phenomenon which Loeb has described in *Strongylocentrotus* and termed "cluster formation," which he interprets with some reserve as a possible tropistic reaction, is also true agglutination, which differs only quantitatively from *Arbacia* and *Nereis*. The conditions under which it occurs, in response to egg secretions of the same species, its character, reversibility, and the specificity of the reaction are identical with *Arbacia*. It is apparently, however, less pronounced, and therefore not so readily recognizable of itself as an agglutination phenomenon. Even the "apparent surface tension phenomena" which Loeb describes for the clusters—"Short streaks or cylinders contract into spherical masses, the above described clusters; and long cylinders break up into a series of small clusters"—are the same as I previously described for *Arbacia* (1913*a*, pp. 550–551).

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[1] Loeb admits that the "cluster formation" exhibits a high degree of specificity. It is therefore inconsistent to interpret the reaction, as he also does, as a "possible tropistic phenomenon" because such phenomena so far as we know do not exhibit specificities of this kind. Agglutination phenomena, on the other hand, as is well known, commonly exhibit equal specificity of a similar kind.

Loeb's interpretation of the "cluster-formation" as a possible tropistic reaction confuses the two sets of phenomena—viz., aggregation (a true tropistic phenomenon) and agglutination—which sperm suspensions may exhibit to the egg-sea-water of its own species. But the aggregation (tropism) can take place only when there is a gradient from the secretion to the spermatozoa. This is realized under the conditions of my experiment of injecting a drop of egg-sea-water into a fresh sperm suspension beneath a raised cover slip; in such a case the two phenomena take place simultaneously viz.; aggregation in the form of a ring around or in the introduced drop (depending on concentration), and agglutination. These two phenomena are produced by two constituents of the egg-sea-water, as I have already maintained.

For the study of the aggregation phenomena therefore it is desirable to employ an agent which has no agglutinative action. This I did in an extensive series of experiments by the method just referred to (1913, p. 533 ff.). To illustrate:—a drop of a $^1/_{100}$ dilution of a saturated solution of CO_2 in sea-water injected into a sperm suspension of *Nereis* in sea-water mounted beneath a raised cover-slip is marked within a few seconds by the formation of a ring of active spermatozoa within the margin of the introduced drop, and separated from the general sperm suspension by a clear zone nearly free of spermatozoa 1.5 to 2 mm. in diameter. I interpreted the ring formation as a positive reaction to the attractive substance (CO_2 and acids generally); the spermatozoa follow the gradient from the suspension into the drop containing CO_2 a certain distance, *i. e.*, up to a certain concentration, and are there arrested. The proof of this interpretation is found in the fact that, if increasing concentrations of CO_2 are used, the ring forms outside the drop and becomes progressively wider, *i. e.*, the migration ceases at a distance from the center which increases with CO_2 or acid concentration (see 1913*a*, pp. 536–538). Loeb suggests that the ring formation with a clear external zone around it is "an indication that the spermatozoa are negatively chemotropic to the strong egg-sea-water, and possibly positively chemotropic to the more diluted egg-sea-water, or to the dense collection of spermatozoa in the ring." The latter suggestion is of course untenable as a primary cause, for the ring-formation is precisely the phenomenon to be explained. It is also unnecessary to assume any negative tropism; the ring formation is due to a limitation of the positive movement by concentration. This is fully discussed in the paper referred to above, but Loeb does not allude to the discussion.

2. The Source of the Agglutinating Substance.

Professor Loeb has also taken issue with me on the question of the origin of the agglutinating substance. He regards his experiments as proving that the substance which causes the "cluster formation" is not formed in the egg but in the chorion; *i. e.*, in the layer of jelly which surrounds the egg. On the other hand I regarded it (and still hold to the opinion) as a secretion of the egg; with which the jelly of course becomes saturated.[1] Loeb's observations again were on *Strongylocentrotus* and mine on *Arbacia*. The issue is a real one even though the chorion is itself a secretion of the egg in earlier stages.

Loeb's conclusion was based on his observation that if the chorion be dissolved off in dilute hydrochloric acid in sea-water, the naked eggs transferred to sea-water produce no detectable amounts of the agglutinating substance any more, whereas the acid sea-water contains it in large quantities. My conclusions were based on the observation that when eggs of *Arbacia* are deprived of jelly (chorion) by shaking, or a prolonged series of washings, they still continue to produce the agglutinating substance in sea-water, though in much diminished quantity; in my full paper, which Loeb had not the opportunity of consulting, I gave series of measurements on this point (1914, pp. 532–538); I also pointed out that in immature ovaries containing many primary ovocytes, but some mature eggs, the quantity of agglutinating substance produced was relatively very small (1914, p. 530), and I therefore suggested that the substance was secreted by the eggs at the time of maturation and was soaked up by the jelly as by a sponge. The eggs, however, continue to produce it after maturation, as I shall show. The immature eggs have as thick a chorion as the mature eggs; therefore the agglutinating substance cannot be merely dissolved chorion. I recognized the possibility of the view expressed by Loeb, investigated it as fully as possible at the time, and rejected it.

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[1] Glaser (1914) also agrees substantially with me: "the agglutinating substance is located in greatest abundance in the jelly and the eggs also contain this material," p. 371.

Since Loeb's paper has appeared, I have repeated his experiments and found my former observations and conclusions confirmed in all respects:

Experiments.—The optimum concentration of HCl for removal of jelly without injury to eggs was found to be 50 c.c. sea-water + 1.4 c.c. N/10 HCl. 1.2 c.c. N/10 HCl in 50 c.c. sea-water did not fully remove the jelly, and 1.6 c.c. caused too much injury to the eggs evidenced by heavy agglutination and later cytolysis. In an experiment of July 17, 1914, the three above concentrations were used. The complete removal of the chorion in the intermediate concentration was demonstrated by observation of the eggs in a thick suspension of India ink in sea-water; even the minutest traces of adherent jelly can readily be detected by this method, but it was all gone. The eggs were then washed as follows: 10.11 A.M. 42/6 c.c.; 10.40 51/5 c.c.; 10.58 50/4 c.c. The supernatant fluid was then tested and found to be free from sperm agglutinating substance; thus furnishing proof that all originally contained in the jelly had been washed out. At 11.20 the supernatant fluid was poured off leaving only 5 c.c. in the tube. The eggs were allowed to settle, and at 11.25 the supernatant fluid was tested and gave a 9–10-second agglutination reaction with fresh sperm suspension. Thus these eggs entirely deprived of jelly by HCl are producing agglutinating substance.

At 4.25 P.M. the eggs were washed again 5/0.7 c.c. and the new fluid gave a 14-second reaction. The next morning the same eggs were washed again 5.5/1 c.c. The new fluid gave a 6–7-second reaction.

These results may be expressed in a different way: thus in an experiment of July 20, a series of eight successive washings of eggs deprived of jelly by acid sea-water represented a dilution of the agglutinating substance contained in the acid sea-water remaining with the eggs of 12,700,800 times. But the acid solvent itself was negative at 1/800 dilution: it was of 400 agglutinating power. In other words, after the removal of the jelly the eggs themselves had produced a sufficient quantity of the agglutinating substance to account for the tremendous difference; and they were still producing it.

These eggs without jelly are fertilizable, as Loeb states, but only 37 per cent. segmented in a heavy insemination of the first day in the experiment of July 17, and only a small part of these developed to the ciliated stage, none of which were normal, most being stereoblastulae and incapable of farther development. The result is entirely similar to that described in my last paper (study VI, '14) for the fertilization of eggs deprived of jelly by shaking and subsequent washing.

The same experiment was repeated on July 18, 20 and 21, with identical results: the eggs from which jelly i[illegible] entirely removed by HCl continue to produce the sperm-agglutinating substance (fertilizin) so long as they live, but their capacity for development after fertilization is much reduced.

In all experiments at least three concentrations of acid were used, and in each experiment it was observed that when the concentration was sufficient to dissolve the jelly there was a good deal of agglutination of the eggs, and in the later washings a great many eggs broke down liberating their pigment. As I have previously shown, broken-down eggs liberate a substance (anti-fertilizin) which neutralizes the sperm agglutinating action of the fertilizin. Therefore, when a sufficient percentage of the eggs are breaking down, the production of sperm-agglutinating substance (fertilizin) by intact eggs may be entirely masked.

I have no intention of disputing Professor Loeb's observations for *Strongylocentrotus*. But they merely prove either that *Strongylocentrotus* sperm is not so delicate an indicator as *Arbacia* sperm, or that the method employed by Loeb was inadequate to detect small quantities of fertilizin. In *Arbacia* the eggs continue to charge the sea-water with sperm-agglutinating substance after complete removal of the jelly, whether by shaking and repeated washings, or by HCl; and the substance continues to be formed as long as the eggs remain fertilizable and living, no matter how often the eggs are washed. *The eggs of Arbacia secrete the substance as I previously maintained. It is not merely the "dissolved chorion."*

It might possibly be objected to this conclusion that the continued appearance of the agglutinating substance in egg suspensions in sea-water after removal of the chorion indicated merely previous adsorption of the substance of the chorion. But the indefinite continuance of its production is inconsistent with the idea of a mere secondary removal of an adsorbed substance. The idea is also inconsistent with the fact that *Nereis* eggs have no jelly at the time laying, but produce a similar sperm agglutinating substance. In this form the jelly also is secreted by the egg after insemination.

Finally if it can be shown that the jelly of immature eggs is entirely devoid of the sperm agglutinating substance, my position that this substance is a later secretion of the egg is rigorously proved. As noted above I maintained the probability of this view in my previous paper (Study VI). This summer my first experiments were undertaken to investigate this point anew.

Fortunately the season was late, and not a single *Arbacia* was ripe when I began work (June 8). This applied to males as well as females: so it was impossible at first to secure ripe sperm as indicator. I therefore made extracts of immature ovaries to be kept for subsequent testing from three females (1, 2, and 3, June 8). June 11 extracts of ovaries in sea-water were made from females 4, 5, and 6: numbers 4 and 6 contained only ovocytes; No. 5 had a large number of ripe ova in addition. On June 16 extracts 1–6 were tested with *Arbacia* sperm suspension: 1, 2, 3, 4, and 6 were absolutely negative; no agglutination. No. 5 gave a strong agglutination reaction lasting about one minute.

It is highly improbable that the agglutinating substance had been destroyed in five of the six, and retained in the only one (No. 5) of the extracts which was made from ovaries containing some ripe ova. So far as these observations go, the jelly of immature ovocytes is free of agglutinating substance.

Again on June 15 I made extracts from ovaries of three females in two of which ripe ova were practically absent, the third had a few. Tested the same day the two former extracts had no sperm agglutinating properties; the third gave slight agglutination.

The females appeared to mature slightly earlier than the males, so that for these experiments I was forced to use rather thin sperm suspensions (mixed more or less with immature spermatozoa), which were probably not as delicate indicators as one could wish. However the difference between the ovaries containing ripe ova and those without was perfectly distinct. Later when fully ripe males could be had all ovaries contained ripe ova.

The following observation also tends in the same direction: June 27, 1914—Three females were selected, of which number 1 was the ripest attainable, the eggs flowing freely out of detached ovaries, and very few ovocytes occur; numbers 2 and 3 were the least mature attainable; number 2 had very few detachable ova, mostly late ovocytes with a sprinkling of ripe eggs; number 3 had quite a few detachable ova with a large proportion of ripe eggs. The ovaries of all three were cut up equally, and sea-water added to each to make 10 c.c. When the ova and ovaries had settled they stood at 1.5 c.c. in 1, at 1.3 c.c. in 2, and 1.5 c.c. in 3. After five hours, tests of the agglutinating strength of the supernatant fluids were made with clear fresh sperm.

No. 1 gave a 10-second reaction at 1/800 dilution.
No. 2 gave a 6-second reaction at 1/10 dilution.
No. 3 gave a 7-second reaction at 1/40 dilution.

Thus No. 1 is 80 times the strength of 2 and 20 times the strength of 3. In general the fertilizin production is proportional to the ripeness of the ovaries.

There is not the slightest doubt in my mind about the demonstrative character of these observations. The appearance of agglutinating substance in the jelly of *Arbacia* eggs is secondary, and takes place probably at the time of breaking down of the germinal vesicle.

Loeb's contention that the agglutinating substance is merely dissolved chorion therefore does not hold for *Arbacia*. With this his argument against my fertilizin theory also falls: "Moreover if it should turn out that the substance which is responsible for the cluster formation is identical with the substance which Lillie calls "fertilizin," which is very likely the case, Lillie's theory becomes untenable, since this substance does not, in all probability, originate from the egg, but from the chorion and since there is, as we have seen, no connection between the presence of this substance and the power of the eggs of being fertilized" (pp. 136–137—Loeb, '14).

In this statement Loeb sums up the essentials of his criticism; since I have shown that "cluster formation" is true agglutination (which Loeb now admits), and that the agglutinating substance (my fertilizin) is not dissolved chorion but a true secretion of the eggs which continues to be produced after the chorion is removed, the entire stated criticism becomes ineffective. There *is* a connection between the presence of this substance and the power of the eggs of being fertilized: the substance can first be demonstrated at the time that the power of being fertilized first arises, viz., after breakdown of the germinal vesicle; it can be demonstrated as long as eggs retain the power of being fertilized, whether the chorion be removed or not, and it disappears absolutely after fertilization, as I showed in my previous paper (study VI, p. 553, 1914).

3. OTHER CRITICISMS.

Another objection raised by Loeb is that "the supernatant sea-water of the eggs of *Strongylocentrotus franciscanus* will not induce cluster formation of the sperm of *Strongylocentrotus purpuratus:* yet the latter sperm fertilizes the eggs of *franciscanus*," from which he argues that the fertilizin of *Strongylocentrotus franciscanus* can not be necessary for the fertilization of its eggs. An error in logic is involved here; agglutination of sperm is merely an indicator of the presence of a certain substance, which is none the less present in *franciscanus* even if *purpuratus* sperm does not reveal it; it *may* nevertheless be activated by *purpuratus* sperm and this is the essential point in the theory.[1] Agglutination of sperm is of no significance except as indicator. As I pointed out in my previous paper, binding of the fertilizin by sperm receptors, *i. e.*, the chemical reaction, is a thing entirely distinct from agglutination; if such binding causes a certain kind of physical surface change of the spermatozoa of suspensions of a certain minimum concentration, they agglutinate; otherwise not. Agglutination is a valuable indicator that enables us to make certain analyses, and that is all. The same principle of fertilization may hold in the entire absence of sperm agglutination.

Another objection in which Loeb supports the possibility of superposing fertilization on parthenogenesis will be dealt with in a separate paper. My contention in this case is that the possibility of such superposition always rests upon incompleteness of the parthenogenetic reaction; if the fertilization reaction be complete, whether by parthenogenesis or insemination, it cannot be repeated. Everybody admits that eggs fertilized by sperm cannot be refertilized; it is a logical impossibility that eggs "fertilized" by parthenogenetic reagents should be refertilized. The problem of the apparent contradiction involved in Loeb's and Herbst's contention of superposition works out in the manner indicated. A study of this problem by one of my students will appear soon.

Loeb cites as a farther difficulty of my fertilizin theory, which he says I have not considered, "that in addition to the membrane forming substance still another, namely a correcting agency, is necessary for causation of the development of the egg." Though

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[1] Loeb states (1914, p. 135): "If the phenomenon of cluster formation were inseparably associated with the power of the eggs of being fertilized, we should expect that sperm should only be able to fertilize the eggs of a species if the egg-sea-water of the same species caused the cluster formation of the sperm." I have never maintained that agglutination ("cluster formation") is inseparably associated with the power of the eggs of being fertilized, but merely that a certain substance produced by the egg is a necessary factor in fertilization. In some cases this substance (fertilizin) produces agglutination of the sperm of its own species, and this reaction furnishes an indicator of its amount, when present, or of its absence. In other cases such an indicator is lacking: I do not find that supernatant sea-water of the eggs of the starfish (*Asteria forbesii*), for instance, agglutinates its own sperm; but I have evidence, to be published elsewhere, that the mechanism of fertilization may be explained in the same way as in *Arbacia*.

I cannot accept this statement of the problem, I have nevertheless taken into consideration the fundamental fact, to which Loeb alludes, in the full account of my experiments, which appeared after Loeb's paper was in press. The fundamental fact is simply that the fertilization process in some cases can be divided in two sharply marked stages. This is perhaps most simply and convincingly shown by my own experiment (Lillie, 1911) of removing the spermatozoon from the egg of *Nereis* after it had already induced the cortical changes, with the result that the developmental phenomena came to a standstill before the first cleavage. I cannot agree with Loeb that the second stage involves a factor corrective of an excess action of the factor of the first stage. I think it is probable that we have a progressive process readily capable of resolution into two stages.

In my complete paper (Lillie, 1914) I considered the second phase of fertilization with reference to the new theory, and may refer the reader to the discussion there given (study VI, pp. 582–584). Here it is only necessary to point out that the "fertilizin" theory is at least as well adapted to account for the two stages as the "lysin" theory.

4. CONCLUSION.

I may be allowed to emphasize the essential features of my theory with some added light thrown by the work of this summer. The fundamental conception is that all agencies initiating development of the egg do so by the same means, viz., activation of an ovogenous substance, which I have termed fertilizin. This conception brings fertilization and parthenogenesis under one conception. I further assumed that such activation in the case of fertilization was caused by union of a constituent substance of the spermatozoon (the sperm receptors) with the fertilizin, and that the activation expressed itself by consequent union of the fertilizin with certain egg substances (the egg receptors). The reaction was thus conceived in terms of the Ehrlich side-chain theory, and was represented diagrammatically accordingly.

That certain chemical combinations form an essential feature of the fertilization reaction cannot be open to doubt. I have not previously taken into account the consideration that the occurence of such reactions, taking place, as they must, across the egg membrane, is dependent on physical conditions of the membrane, especially its permeability to the substances concerned. In speaking, as I did, of five blocks to the fertilization reaction, I was concerned only with the chemical reactions involved. There may be other blocks of a physical nature. Indeed these were much in evidence in the fertilization of *Asterias*, which I studied in the first part of the summer, and shall report on elsewhere. Another important consideration is that the reaction must also be dependent on environmental conditions such as temperature, ionic constitution of the medium (see Loeb, '14*b*), etc. Blocking of fertilization may also arise from such causes.

Continuing the exposition of the theory; I identified the fertilizin of *Arbacia* with the substance found in the fluid of egg suspensions which causes agglutination of sperm suspensions of the same species. This phenomenon cannot possibly be lacking in significance, for it furnishes direct evidence of a combination of egg and sperm derivatives; the phenomenon itself is not concerned in fertilization, for a single spermatozoon may fertilize an egg. Neither does the absence of such agglutination in other species affect in the least the conclusion that may be drawn from *Arbacia:* because we may have a combination of egg and sperm derivatives without any sperm agglutination. The agglutination is incidental, the combination is the essential thing.

The fertilizin theory in its essential aspects is not dependent on the identification of fertilizin and sperm agglutinating substance. I believe in their identity; but if it were proved, as Loeb has sought unsuccessfully to do, that the agglutinating substance is not essential for fertilization, the fertilizin theory would still not be attacked in its essence. The conception that initiation of development is essentially a phenomenon of activation would still stand in opposition to theories of external agents acting directly by corrosion (cytolysis), or coagulation, or what not. The egg could still be regarded as a self-contained system with no more than the usual environmental relations. It is only from this point of view that the complex phenomena of parthenogenesis and fertilization can be united in a logical whole.

The theory of the identity of fertilizin and sperm agglutinating substance rests upon a considerable body of ascertained facts (see study VI), and it gives us at once a point of attack and a working hypothesis of considerable value. I have been able to show for instance that the origin of the capacity of the egg for being fertilized can be understood on this basis; that the cessation of fertilization capacity can also be so understood; and that the physiological sterility (prevention of polyspermy) of fertilized eggs is readily explained by the neutralization of the fertilizin by a substance (anti-fertilizin) demonstrably present in the egg.

On the other hand the theory does not postulate that the fertilizin of all forms should agglutinate sperm of its own species. There may be many forms in which the union of the sperm receptors with fertilizin does not produce such physical changes of the spermatozoa as to lead to agglutination. In those cases in which agglutination does occur we have a reaction very useful in analysis; but it cannot be too strongly emphasized that the agglutination itself is to be regarded merely as an indicator of the essential reaction.

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OBSERVATIONS ON THE SPERMATOGENESIS OF THE GALL-FLY, DRYOPHANTA ERINACEI (MAYR).

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INTRODUCTION.

Dryophanta erinacei is one of the gall-producing *Hymenoptera* having two generations in the year: males and females in the spring, and females alone in the fall. The fertilized eggs of the bisexual generation produce females exclusively, while the unfertilized eggs of the female generation produce both males and females.

The material for this study was obtained on April 13, 1914, from galls occurring on white oak trees (*Quercus alba*) in the neighborhood of Cincinnati. The galls are smooth spherical protuberances on the bud-scales, several millimeters in diameter. Usually one, but occasionally two, and sometimes three galls very close together are found at the end of a single twig. Twigs bearing galls were cut off, brought into the laboratory, and the cut ends inserted in sand moistened with water. Ten days later males and females emerged from the galls, and continued emerging for two weeks. Copulation took place immediately after emergence.

These galls supplied all stages of developing males and females from the late larva to the imago. For fixation Petrunkewitsch's fluid was used; penetration being facilitated by making a longitudinal incision through the body wall. In some cases ovaries and testes were dissected out, but better results were obtained when the organs were left in situ and the entire animal sectioned.[1]

Embedding was done in rubber-paraffin, and serial sections cut 10 μ in thickness. Two methods of staining were used: safranin and light-green, and iron-haematoxylin with or without counterstain. After dehydration the stained sections were cleared and mounted in euparal. Euparal offers several advantages over balsam as a mounting medium. Thus its use obviates running stained sections through absolute alcohol; since sections may be transferred directly from 95 per cent. alcohol to euparal. Next the index of refraction of euparal is low 1.483. And lastly euparal dries quickly, so that sections may be studied at the end of twelve to twenty-four hours after mounting, without danger of injury.

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[1] For assistance in collecting and preserving material I wish to acknowledge my indebtedness to Dr. Annette F. Braun.

Observations.

The testes of the late larva and early pupa show primary spermatocytes at the end of the growth period as large polygonal cells having a reticulated nucleus containing a poorly defined nucleolus, often of a bipartite character (Fig. 1). The nucleolus does not take the safranin stain as deeply as the chromosomes, and thus differs markedly from the chromosome nucleolus of the primary spermatocytes of many *Hemiptera.*

A true primary spermatocyte division does not occur. Instead, a small mass of cytoplasm free of chromatin is constricted off, forming the so-called polar body. Preparation for this suppressed or abortive division begins with a change in the outline of the cell, the spermatocyte assuming a pear shape (Figs. 2, 3 and 4). From the narrow end of the cell and forming the stem of the pear, extends a short filar process. At the base of this process, which at first glance suggests the tail of a spermatozoon, is often found a light basic-staining spherule which may or may not be a centrosome. While these changes are taking place in the cytoplasm the nucleus undergoes a slight contraction and the chromatin passes through a series of transformations terminating in the formation of chromosomes (Fig. 4).

The next step in the process is somewhat uncertain and there may be some question as to seriation. It seems that after the chromosomes are completely formed, they become massed in clumps at one side of the nucleus, and from these masses distinct loops extend toward the opposite side of the nucleus (Figs. 5 and 6). The cell shortens, the filar process becomes less distinct (Fig. 6), and a portion of the cytoplasm is constricted off (Figs. 7 and 8). As this is taking place the nuclear membrane appears very irregular in outline but seems to remain intact. Inside the nuclear area the chromosomes are in the form of single rods whose free ends extend toward the polar body. There is every appearance to indicate a resistance of the part of the chromosomes against a tension pulling toward the polar body. Distinct spindle fibers are not to be seen, but the cytoplasm contains a reticular structure which may represent a poorly developed spindle. The polar body is quickly cut off from the cell to which, however, it may remain attached for a considerable length of time (Fig. 15). The free polar body of Fig. 8 belongs to a cell in an adjacent section. Polar bodies cut in various planes are frequently seen in the spaces between spermatocytes at this time (Figs. 8 and 16) and throughout the second spermatocyte division. The complete absence of polar bodies in cysts containing cells with the chromosomes in the looped condition of Figs. 5 and 6 makes it almost certain that the looped stage precedes that of Figs. 7 and 8, in which the chromosomes show free ends.

Preparations for the second spermatocyte division follow very rapidly. After the formation of the polar body, the second spermatocyte rounds up; the knot of chromosomes separates into distinct, short, thick, curved rods, 12 in number (Fig. 9). In the cell figured here, a late prophase, the nuclear membrane is fairly distinct. Details of spindle formation were not observed. Figs. 10 and 11 show characteristic side-views of spindles at metaphase. The chromosomes seldom lie in one plane so that counting even in polar views is a difficult matter. In such views, as in Figs. 12 and 13, 12 chromosomes can be counted with considerable accuracy in the majority of cases.

A characteristic late telophase is shown in Fig. 14 which resembles to a striking degree a somatic mitosis, and strongly suggests that the chromosomes have been divided longitudinally. In later stages of this division (Fig. 15) the chromosomes become packed into dense compact masses, so that it is impossible to determine the number of constituent chromosomes in the daughter groups. When reconstitution of the nuclei occurs (Fig. 16), these masses break up into slightly bent rods of ragged outline. In cross section these rods appear as dots of which 12 can often be counted. Counts of the daughter groups of chromosomes made in this way are not very satisfactory, since one is never sure that a cross-section includes all of the rods or that a single rod has not been cut more than once.

The spermatids formed by this division seem therefore to be equal in size and chromatin content, and all of them develop into spermatozoa. There is no evidence of a heterochromosome or chromatoid body passing undivided into one of the spermatids.

By the end of the second spermatocyte division all of the polar bodies are detached and show signs of disintegration, fragments being frequently seen in the intercellular spaces giving the appearance shown in Fig. 16.

The relatively distinct outline of the chromosomes seen in this last figure persists for but a short time and is completely lost in the young spermatids. Figs. 17 and 18 are early stages in the transformation of the spermatids into spermatozoa.

Such in brief is an outline of the main features of development of the germ cells in the male of *Dryophanta* from the growth period to the spermatids. There is but one true maturation division—that of the second spermatocyte. The first spermatocyte division is indicated by the pinching off of a small quantity of chromatin-free cytoplasm which forms the so-called polar body.

Discussion.

Doncaster in his studies of the gametogenesis of the gall-fly, *Neuroterus lenticularis*, arrived at certain conclusions which may be considered at this point. This species of *Hymenoptera* has a similar life-history to that of *Dryophanta*. Thus according to Doncaster the female generation emerges in April from galls formed during the preceding summer and immediately lays eggs in oak buds (species?). Early in summer the galls appear from which males and females emerge. After copulation the female lays eggs in the tissues of young leaves at the side of a small vein. From the galls resulting, females emerge in the following spring.

As in *Dryophanta*, therefore, the fertilized eggs of the bisexual generation develop into females; while the unfertilized eggs of the female generation produce both males and females.

Doncaster found that the first spermatocyte division is abortive—a small portion of the cytoplasm being constricted off as the polar body. This is followed by a resting stage which resembles the metaphase of a true division, but is distinguished from it by the persisting nuclear membrane and the position of the chromosomes at one end of the nucleus near the broad end of the cell. No nuclear division takes place but the nucleus becomes oval in shape and the chromosomes generally contract to form a compact mass lying across its center. In some cells at least this chromatin mass seems to divide—one half passing to each side of the oval nucleus. The chromatin may finally disperse and give rise to a condition resembling the first spermatocyte in which the chromatin has begun to appear. "Possibly the division of the chromatin inside the nucleus, which occasionally seems to occur, is the persistent remnant of a true nuclear division, or it may be compared with the 'intranuclear karyokinesis' described by Kostanecki in the parthenogenetic eggs of *Mactra*" (p. 93). Toward the end of the rest stage the chromatin becomes grouped in the form of large elongate granules or small bands having a more or less meridional arrangement under the membrane.

The second spermatocyte division in *Neuroterus* is a true mitotic division in which the haploid number of chromosomes, 10, appears on the spindle to be equally divided between the daughter cells. There is also a small stained body lying outside of the spindle which passes undivided to one of the spermatids.

In the spermatogonia and in mitotic figures of nerve cells in the developing nervous system Doncaster finds the halpoid number of chromosomes, 10, but in mitoses of immigrant mesoderm cells the diploid number, 20.

The eggs layed by the females of the bisexual generation undergo two maturation divisions; leaving 10 chromosomes for the female pronucleus. The spermatozoon brings into the egg 10 chromosomes, and 20 chromosomes appear on the cleavage spindles. The parthenogenetic eggs of the female generation may be divided into two groups: Those which undergo maturation and develop into males; those which omit the maturation divisions and develop into females. In the first group 10 chromosomes are found in the cleavage divisions; in the second group 20. Since any female produces only one kind of egg, there are male-producing females and female-producing females.

Mitoses in the nervous system of all females show the diploid number of chromosomes.

Returning now to *Dryophanta* I should like first to consider the stage represented in Figs. 5 and 6, which I believe corresponds to the second spermatocyte resting stage mentioned by Doncaster in *Neuroterus*. The figures at first glance suggest the synapsis stage of other insects, but in view of other facts it is difficult to interpret the condition as a fusion of chromosomes. Earlier stages such as the prophase shown in Fig. 4 display the same number of chromosomes as appears in the second spermatocyte division, 12, which is assumed to be the haploid number approximately. Since there is no evidence in *Dryophanta* of an intra-nuclear division of these 12 chromosomes into two groups, a true synapsis at this time would be equivalent to a second "reduction." A more probable interpretation of this "looped stage" and one that is warranted by a close study of the sections is that the limbs of a loop are the halves of a chromosome that has undergone a temporary and incomplete splitting. With the next step in the process, the formation of the polar body, the split disappears and the chromosomes have every appearance of being single, solid rods (Figs. 7 and 8). The latter condition might of course be brought about by breaking of the loops at the middle, but in that event one would expect to find twice as many single chromosomes as loops. Such is not the case, for the number of unsplit chromosomes is the same as the number of loops so far as could be determined. Reversing the seriation at this point would of course change the interpretation offered here; but the main reason for placing the looped stage before the other, as has been mentioned above, is that there is no evidence of polar body formation at this time. And to this may be added the fact that the outline of the cell at the looped stage as shown in Fig. 6 represents an intermediate condition between that of Fig. 4 in which there can be no question about polar bodies being absent, and Figs. 7 and 8, in which the polar bodies certainly are present.

An actual resting stage, if one occurs at this time, must be of very short duration. The second spermatocyte division follows very quickly after the formation of the polar body. Fig. 9 represents a prophase of this division in which the chromosomes are surrounded by an intact nuclear membrane. The spindle area of the second spermatocyte is rather distinctly marked off from the rest of the cytoplasm (Fig. 10) and suggests that the nuclear membrane disappears very slowly.

Polar views of the metaphase display, as nearly as could be determined, 12 chromosomes, presumably the haploid number. It would seem that each chromosome is divided quantitatively by a longitudinal splitting; although it must be remembered that attempts at verifying this conclusion by studying the constituents of the daughter groups are not satisfactory owing to the tangled condition of the chromosomes.

I find nothing resembling the small stained body which in *Neuroterus* according to Doncaster passes undivided to one of the spermatids. As Wilson has observed this body is of the same nature as the chromatoid body seen in the growth-period and spermatocyte-division of *Pentatoma*. The chromatoid body is of rounded form, dense and homogeneous consistency, and after double staining with haematoxylin or safranin and light green is at every stage colored intensely blue-black or brilliant red, precisely like the chromosomes of the division period or the chromosome-nucleoli of the growth period. Nevertheless Wilson finds that the body is neither a chromosome nor any kind of a chromosome and takes no visible part in the formation of the spermatozoa. In the transformation of the spermatids it wanders far into the sperm-tail and is at last cast off altogether.

I have not yet had opportunity to study the maturation phenomena of the egg in either generation of *Dryophanta*, but observations confined to individuals of the bisexual generation point to general conclusions which differ somewhat from Doncaster's views regarding the chromosomal relations in the alternate generations. In the material at my disposal spermatogonial divisions are not abundant enough to determine the number of chromsomes. While mitoses abound in the somatic cells of male larvae and pupae, it is difficult to find good clear metaphases; but wherever counts were possible, the number found was 12 (Fig. 19). In the follicle cells of the ovary I have found it less difficult to count the chromosomes. Figs. 20, 21 and 22 are drawings of metaphase plates of such cells in which the numbers are respectively 13, 14 and 13.

In the somatic cells of both males and females one occasionally finds mitotic figures containing a much larger number of chromosomes, but such cases are in the nature of exceptions and no one would contend that they represent an average condition. If there is such a thing as constancy in the number of chromosomes in the majority of somatic cells, the constant is in the neighborhood of 12 in both males and females of the bisexual generation. Because this is the number of chromosomes found in the second spermatocyte division, 12 is assumed to be the approximate haploid number. Now in any case where an accurate count is difficult or impossible in the somatic cells, it is always possible to determine with certainty that the number is very much less than the expected diploid number 24. In view of the fact, that in the honey-bee it is said that the somatic mitoses show a very much higher number of chromosomes than occurs in the gonial cells, somatic mitoses should not be used as a safe and reliable method of determining the diploid number. There may however be some significance in the fact that a large number of somatic cells of both males and females of *Dryophanta* contain a number of chromosomes that approximates the number found in the dividing spermatocyte rather than a multiple of this number.

Any definite statement regarding the origin and significance of this condition must await examination of the maturation and cleavage spindles of the egg. However, the facts at hand do suggest that the males and females of the bisexual generation of *Dryophanta* develop from eggs whose chromosomes have undergone reduction in maturation. The slightly large number of chromosomes found in the females somatic tissues may or may not be of significance, but if sex determination has its basis in the chromosomes, a difference in the method of distribution of the chromosomes in maturation may explain why some of these eggs develop parthenogenetically into females and others into males.

In a recent paper Nachsheim has summed up in a general statement the results of investigations dealing with sex-determination in *Hymenoptera* as follows: "Die Männchen der Hymenopteren entstehen aus unbefruchtete Eiern, die zwei

Richtungskörper abgeschnürt und eine Reduktion ihrer Chromosomenzahl erfahren haben. Sie besitzen also nur ein Chromosomensortiment, das mütterliche, und infolgedessen muss in der Spermatogenese die Reduktionsteilung unterbleiben. Die Weibchen der Hymenopteren besitzen beide Chromosomensortimente, also die diploide Chromosomenzahl in ihren somatischen Zellen, da sie aus befruchteten Eiern ihre Entstehung nehmen oder—bei den Blatt- und Gallwespen—zwar ebenfalls aus unbefruchteten Eiern, aber aus solchen, die den Reifungsteilungen ihre Chromosomenzahl nicht reduciert haben; entweder findet in diesen Eiern überhaupt nur eine Reifungsteilung statt, oder beide Reifungsteilungen sind Aquationsteilungen. Der zweite Richtungskörper kann also. . . . an Stelle der Spermatozoons treten, d.h. der zweite Richtungskörper bringt in Verbindung mit der Eikern dasselbe Geschlecht hervor wie der Eikern in Verbindung mit einem Spermakern" (pp. 220–221).

My findings in the somatic chromosomes of *Dryophanta* raises the question as to whether females of the bisexual generation are produced parthenogenetically from eggs that do not undergo reduction in maturation. An examination of maturation stages in the egg is necessary to decide this point and material for this purpose is being collected at the present time.

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EXPLANATION OF PLATES.

The figures are camera drawings made at table level with Zeiss apochromatic objective, 1.5 mm. and compensating ocular, 12. There has been some reduction in reproduction.

Plate I.

Fig. 1. Primary spermatocyte at the end of the growth period. Male pupa.

Figs. 2, 3 and 4. Primary spermatocytes undergoing changes in outline preliminary to the formation of the polar body.

Figs. 5 and 6. Primary spermatocytes having chromosomes in the form of loops or split rods.

Figs. 7 and 8. Stages in the cutting off of the polar body. Fig. 8 contains a second polar body belonging to a cell in a neighboring section.

Fig. 9. Prophase of the second spermatocyte division showing 12 chromosomes.

Fig. 10. Side view of the second spermatocyte spindle at metaphase.

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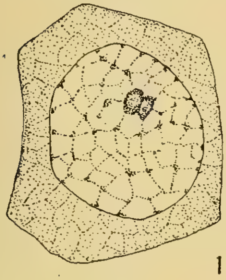
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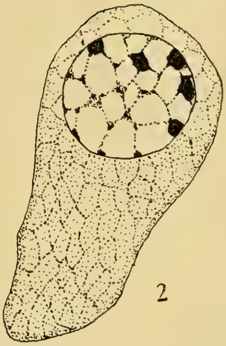
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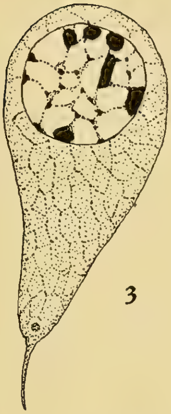
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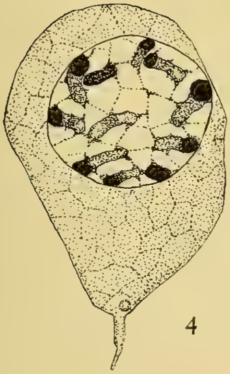
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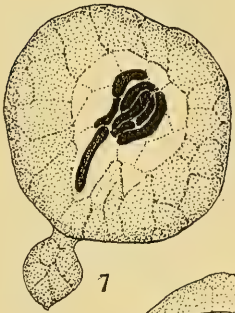
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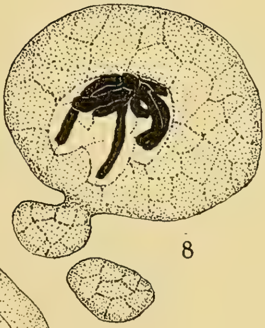
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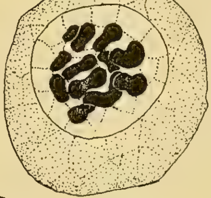
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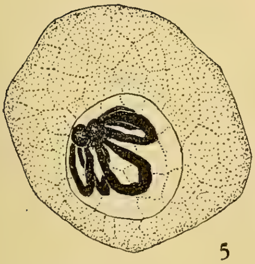
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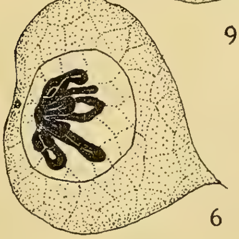
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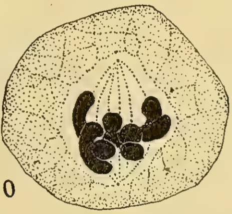
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H. L. WIEMAN.

Plate II.

Fig. 11. Side view of the second spermatocyte spindle at metaphase.

Figs. 12 and 13. Polar views of the second spermatocyte spindle at metaphase showing 12 chromosomes.

Fig. 14. Second spermatocyte spindle at late anaphase showing a free polar body near the upper end of the cell.

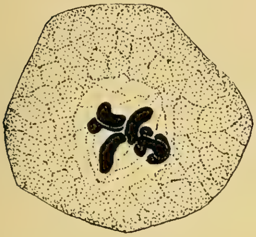
Fig. 15. Second spermatocyte at telophase with a polar body attached to the upper daughter cell.

Fig. 16. Early spermatid, reconstruction of the nuclei. Polar body fragments near the upper cell.

Figs. 17 and 18. Stage in the transformation of spermatids into spermatozoa.

Fig. 19. Metaphase chromosome group in the mitosis of a developing wing, showing 12 chromosomes. Young male pupa.

Figs. 20, 21 and 22. Metaphase chromosome groups of ovarian follicle cells, showing 13, 14 and 13 chromosomes respectively. Late female larva.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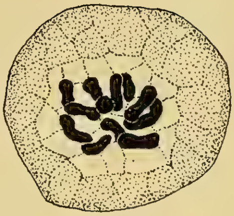
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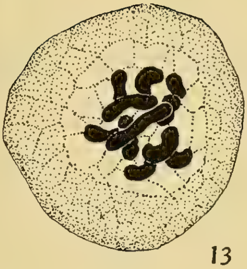
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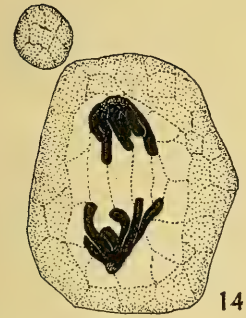
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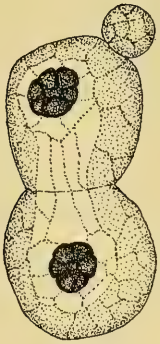
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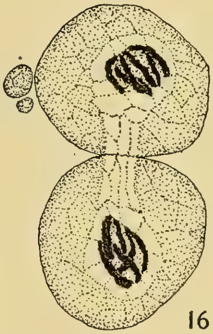
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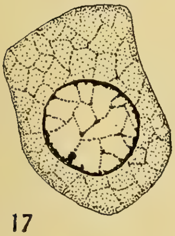
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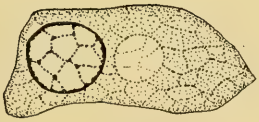
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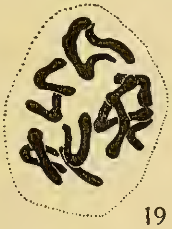
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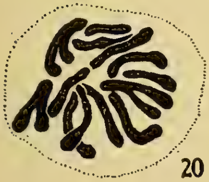
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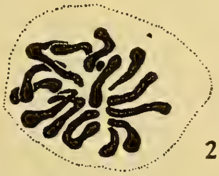
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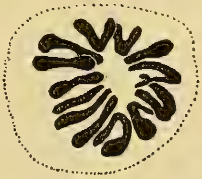
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H. L. WIEMAN.

EGG ALBUMEN AS A CULTURE MEDIUM FOR CHICK TISSUE.

OLIVE SWEZY.

Egg albumen as a culture medium for chick tissue *in vitro* has received but scant attention from experimentalists, in spite of the fact that it forms the natural medium, in part at least, of the embryo chick. In a recent series of experiments, however, results have been obtained which show that all the usual manifestations of cell activity, noted by various observers in other culture media, were to be met with in cultures made from egg albumen, and have, I believe, demonstrated satisfactorily its entire adaptability to that use. These experiments were carried on in the laboratory of Prof. S. J. Holmes, to whom my thanks are due for his kindness in giving advice and assistance throughout the course of the work.

The technique followed has been that outlined by Burrows and Carrel, modified to suit the different conditions under which the work had to be carried on, using embryos varying in age from twenty-four hours to fourteen days. Of these it was found that the most successful results were obtained from embryos of from ten to fourteen days growth, though all showed considerable activity. Fragments of all the organs of the body, including the brain and spinal cord, were used, but the most active growths were obtained from the heart. Several series of preparations were made by cutting up the entire embryo into minute particles in a small amount of Ringer's solution and egg albumen, stirring and shaking these rapidly for a few minutes and then placing a small drop of the mixture on the slide and sealing in the usual way. By this process cultures could be made containing but a few or even single cells. The medium used has been egg albumen alone or mixed with varying proportions of egg yolk, Ringer's solution and extract of muscle tissue. Egg yolk proved entirely unsatisfactory because of the impossibility of seeing what was taking place within it. The best results were obtained from egg albumen alone and with mixtures of albumen and muscle tissue extract, the latter being prepared from embryo chick tissue and added to the albumen either before or after making the culture. Egg albumen coagulates to a more or less firm consistency and thus gives one of the conditions apparently requisite for the growth and activity of the tissue cells.

Owing to the viscosity of the albumen, considerable care is necessary in handling the specimens when it becomes needful to transfer the culture to a fresh medium, the usual method of procedure being to cut away the old albumen with a sharp knife. When, as is frequently the case, the outgrowth seemed to be mainly on the surface of the glass, and thus could not be transferred in the usual way without the loss of the greater part of the growth, another method was used. Inverting the cover glass the albumen was removed with forceps and pipette, several changes of Ringer's solution successively placed over the culture and, after removal of this, a fresh drop of albumen was added to the culture and it was again sealed up.

The latent period, before the beginning of activity of the culture, lasted from half an hour to several days. Usually, in good preparation, active amœboid movements began within half an hour after being put on the slide. At that time along the border of the tissue could be seen the elongated, outpushing cells forming a fringe along what was before a clear cut outline, with a few scattered cells lying at some little distance from the main mass. These cells displayed very active amœboid movements that are less common in the older cultures though still present to some extent. When these cells are chilled or disturbed they contract and become rounded. On a number of cultures groups of cells showed long clear processes extending outward, sometimes branched, with the ends breaking up into short filaments. These were in all cases cultures which included portions of the brain or spinal cord from a four-day chick. An attempt was made to photograph one of these cultures but the length of time necessary was sufficient to chill the slide and, on examination, it was found that the processes had all been retracted. Subsequent incubation had no effect on the culture, though disintegration did not take place for several days. In all the cultures these processes disappeared, were retracted apparently, in the course of fifty to seventy hours and no further evidences of them were seen. In the preparations made by shaking up the finely cut embryo with Ringer's solution, a greater or less number of single cells were found. In the course of a few days these were greatly increased in number with a distinct massing together of the cells, usually along the outer border of the drop of albumen. Owing to accidents of various kinds these were not carried along far enough to show the tissue formation noted by Carrel.

The most marked instance of tissue formation was that apparent in a culture made from the heart of a fourteen day chick, which, at the end of twenty days was encircled by a new formation five times the diameter of the original piece of tissue. This new formation was several cells in thickness and composed of fusiform and polygonal cells, sometimes massed together, forming a network, or in other places showing distinct cell boundaries. Among these cells many showed division figures at various stages. Around the outer margin of the mass of cells and extending nearly three-fourths of the entire distance around it, the cells had taken on a different character. Here they had become flat, thin and elongated in a direction parallel with the margin of the circle. This formation was several cells in thickness with the cells closely matted together and forming a distinct boundary that was conspicuous without the aid of a lens. The remaining one-fourth of the margin was occupied by cells actively pushing outward.

To test the effects of cold on the growth of the tissues, the embryo was sealed up in a stender dish containing Ringer's solution and placed in the ice box of the refrigerator with the temperature but a few degrees above zero, Centigrade. The first of these was used the second day and behaved like normal tissue. Most of those kept in the refrigerator for a number of days became infected with bacteria. The longest period of cold storage which gave successful cultures was four days, from January 31 to February 4. One half hour after making the cultures from this embryo the cells were moving out in an active condition in four out of the sixteen cultures made. The subsequent history of these cultures was the same as that of unrefrigerated tissue.

The longest period during which tissues have been kept alive without any evidences of necrobiosis has been ninety-three days, and in the majority of these cases death has been caused by infection with bacteria or molds or other accidents, and, not, apparently, by any lack of vigor in the tissues themselves. This, in general, seems to be true of most of the cultures which appear to be in a thriving condition after the second day or third day, and especially where renewals of the culture medium have been frequent, and precautions have been taken to avoid tearing or otherwise injuring the tissues. However disintegration frequently takes place from no apparent cause.

Egg albumen presents some difficulties when a stained preparation from the culture is desired, on account of its avidity for stains. In the first stained preparations made it was impossible to distinguish the outlines of the cells, and the study of the specimen seemed a hopeless task. This difficulty was later overcome by the following methods: the cover glass was inverted and placed on the mouth of a vial containing a quantity of osmic acid. The mouth of the vial was small enough to be completely covered by the cover glass and yet not touch the preparation. After fixing in this manner for ten minutes the cover glass was placed in a stender dish containing distilled water and left for a number of hours. Frequent agitation and changes of the water removes the greater part of the albumen, leaving the tissue adhering to the glass, which may then be put through the alcohols and stained in the usual way. With this method very clear preparations may be obtained.

Zoölogical Laboratory,
University of California,
Berkeley, Cal., October 13, 1914.

THE INFLUENCE OF PRODUCTS OF PATHOLOGIC METABOLISM ON THE DEVELOPING TELEOST OVUM.

E. I. WERBER,

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In his recent work on pathological human ova, after careful sifting of anatomical evidence, Mall[1] arrives at the conclusion that the failure of large numbers of ova to develop normally is to be traced to diseases of the uterus. According to his view, which is supported by obstetrical and gynecological data, diseases of the uterus are the primary cause of the faulty implantation of the ovum. This in turn makes proper nutrition of the developing embryo impossible thus leading to various degrees of malformations by arresting development. The deformed embryo is eventually aborted after it has exhausted its inadequate means of subsistence in the uterus. Full-term monsters would be born from such deformed embryos if they were not hindered in their further development by starvation. According to this theory, therefore, an apparently healthy ovum discharged into a diseased uterus fails to develop normally owing to its defective implantation.

Mall studied largely pathological ova of the first two months and the interpretation of the numerous cases described by him seems justified. Practically all pathological ova of the early months studied by him as well as by other investigators, exhibited the condition of faulty implantation, so that it is not unwarranted to regard this condition as the direct cause of monstrous development.

A consideration of some instances of arrested, defective or even monstrous development found after full-term birth would suggest, however, that there must be also some other factors which primarily interfere with normal development. Such defects as rudimentary development of one or both eyes, congenital absence of both arms, hydrocephalus, possibly also cases of congenital deafness, to mention only a few that are well known to occur, can, in the writer's opinion, hardly be traced to defective implantation. The results of investigations in experimental teratology by Panum,[1] Dareste[2] and more recently by Stockard[3] and Bardeen[4] would seem to suggest that some physico-chemical factors may be at work in a great number of cases of pathological development. These factors may in some instances be the primary cause of terata, while in other cases they may be only secondary contributing causes.

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[1] Mall, F. P., "A Study of the Causes Underlying the Origin of Human Monsters." *Journ. of Morphology*, Vol. XIX., 1908; "The Pathology of the Human Ovum" in Keibel-Mall "Handbook of Human Embryology," 1910.

The experimental teratologists subjected developing ova in very early stages to changes in the physico-chemical nature of the environment and found that various monstrosities could be produced under these conditions. It was impossible, however, for them to control the results of experimentation, as they could not predict the type of monster which would result from the employment of the same factors. The experiments of Stockard, where a more or less definite monstrosity—cyclopia or monophthalmia—appeared with considerable certainty in a large percentage of embryos developing in magnesium chloride or alcohol solutions, mark a distinct progress in this field of inquiry, because they paved the way towards experimental control of monstrosities occurring in nature.

To the writer Stockard's work suggested the possibility that the monstrosities met with in higher animals and man may to a certain extent be due to the influence of injurious substances found in the circulation under pathological conditions. While this hypothesis could not be applied to bacterial toxins on account of insufficient knowledge, it seemed that some substances thrown into the circulation in various metabolic diseases may be responsible for pathological development. Thus the etiology of defective or monstrous development would be traced to the pathological metabolism of the mother or possibly even of the father. For, as Bardeen[1] has shown, a normal, healthy ovum of the toad, if fertilized with sperm which had been injured by exposure to the action of X-rays, will give rise to a deformed embryo.

---

[1] Panum, "Entstehung der Missbildungen," 1860.

[2] Dareste, "Recherches sur la production de monstrosites," Paris, 1891.

[3] Stockard, C. R., "The Artificial Production of a Single Median Cyclopean Eye in the Fish Embryo by Means of Seawater Solutions of Magnesium Chlorid," *Arch. f. Entwmech.*, Vol. XXII., 1907; "The Influence of Alcohol and Other Anæsthetics on Embryonic Development," *Am. Jour. of Anat.*, Vol. X., 1910.

[4] Bardeen, C. R., *Jour. of Experimental Zool.*, 1907; *Am. Jour. of Anat.*, Vol. XI.

With this idea in mind the writer conducted during the summer of 1914 experiments on eggs of *Fundulus heteroclitus*. The eggs of this fish are easily obtained at Woods Hole and are excellent material for experimentation. The investigations on the fish eggs are of a preliminary character, and were undertaken to ascertain the influence of some toxic substances occurring in pathological metabolism on the developing egg.

The number of these substances being rather large while the spawning season is limited to a few weeks, it was impossible to try more than a few of the chemicals. Urea, butyric acid, lactic acid, sodium glycocholate, acetone and ammonium hydroxide were tried as to their effect on the development of fertilized eggs. Definite results were so far obtained only with butyric acid and acetone.

Ten c.c. of a 1/12–1/14 molecular solution in 50 c.c. of sea water was found to give the greatest number of monsters when butyric acid was used. The eggs were submitted to the action of this solution for 20 hours after they had reached the eight-cell or sixteen-cell stage, *i. e.*, 3 to 3½ hours after fertilization. While under this procedure numerous monstrosities were at first obtained, the method failed almost completely in later experiments. I therefore employed developing eggs in the first stages of division (2- and 4-cell stages) when many monstrosities were produced even after a sojourn of thirty hours in the butyric acid solution. But it seems to me that the reason why the method failed with the eggs in more advanced cleavage stages was that the time of exposure was too long, as very many eggs were dead by the end of that treatment, and that with an exposure of 10 or 15 hours better results would have been obtained.

There is, however, as important difference in the effect which this toxic substance has upon developing eggs in the first and second or in the third and fourth divisions. In the former case anterior hemiembryos, dwarf embryos with deformities of the eyes or of the otic vesicle, and malformations of the most extreme kind were predominant, while in the latter deformities of the eye such as cyclopia and monophthalmia, etc., were mostly observed. In either case, however, there were very few embryos in which only the nervous system was affected. In most of the deformed embryos all organ systems were more or less involved in the malformation.

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[1] Bardeen, C. R., *Jour. of Experimental Zool.*, 1907; *Am. Jour. of Anat.*, Vol. XI.

Similar results were obtained with acetone in sea water, varying in concentration from 20–50 c.c. of a molecular solution in 50 c.c. of sea water. In this mixture the eggs remained from 24–72 hours from the eight-cell or sixteen-cell stage. In every case great numbers[1] of monsters similar to those already mentioned were produced.

The monstrosities in both series of experiments with butyric acid and acetone being essentially alike it will not be necessary to describe separately the deformities produced by each.

Cyclopia and asymmetric monophthalmia were found to occur rather abundantly. There were also some cases of asymmetric monopththalmia in which an open orbit was found on the side lacking the eye. It is of some interest to note in this connection that the eyeless orbit in such cases is usually closed on the outside by periorbital tissues. The anatomy of the head of such embryos may probably reveal some interesting conditions. Other cases of asymmetric monophthalmia were found in which an apparently free eye had developed on the yolk-sac at a considerable distance from the embryo. Probably the most striking of the results obtained in this investigation were some eggs in which nothing could be observed but an eye. In only one case this eye seemed to be perfectly developed, while the other solitary eyes had "coloboma"-defects, the fissure of the chorioid still being patent. Only a few (five or six) of these malformations are recorded, but in spite of their rare occurrence they are very significant from the standpoint of experimental embryology. At the present time it is, obviously, impossible to account for the occurrence of these remarkable cases. However, it is hoped that an anatomical investigation of early stages in the development of eggs subjected to the influence of the environmental modifications used in these experiments, may give at least a clue as to what may have happened in the development of these eggs. Practically all other known deformities of the eye such as total blindness, or presence of lenses only, or presence of supernumerary lenses were frequently found.

---

[1] No attempt was made to ascertain the percentage of the deformities found in these experiments, this part of the work being deferred to later investigation.

To the student of the physiology of development the occurrence in these experiments of large numbers of anterior hemiembryos which seem to be closely analogous to those obtained by mechanical means by Roux,[1] Endres,[2] Morgan[3] and K. Ziegler[4] will be of special interest. As will be pointed out soon the formation of the hemiembryos in these experiments may also possibly be due to similar factors.

A great number of embryos were hydrocephalic and so far as could be determined it is reasonable to expect that an anatomical investigation may reveal in some deformed embryos oedematous conditions, also herniae and other mechanical obstructions which played a part in their formation.

Striking abnormalities of the heart and blood-vascular system were found in all malformed embryos with the exception of those which showed only median cyclopia. Some were entirely devoid of the heart, while other possessed an exceedingly delicate tube in its place which was practically straight and of about the size of the intestinal blood vessels in a normal embryo of a corresponding stage. The rate of the heart beat varies with the degree of the abnormality of the organ, and is, as a rule, very slow in all monstrous embryos. The range of variation in the development of the blood vessels is very wide. There may be merely blood islands scattered on the yolk-sac, rudimentary, imperfectly connected, or in some instances more or less normal vessels.

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[1] Roux, W., "Gesammelte Abhandlungen zur Entwicklungsmechanik der Organismen," II., 1895.

[2] Endres, H., "Anstichversuche an Froscheiern," *Sitzber. d. zool.-bot. Sektion d. schlesischen Ges. f. vaterländische Kultur*, 1894.

[3] Morgan, T. H., "The Formation of the Embryo of the Frog," *Anat. Anz.*, 1894.

[4] Ziegler, K., "Zur Postgenerationsfrage," *Anatomische Hefte*, Vol. LXVI., 1902.

Twins were found only in a few cases and only once were true "Siamese" twins observed. They were much deformed, had one common heart and only vestigial eyes. Several eggs were recorded, in which an anterior duplicity had developed. In one of these latter cases the components of the duplicity were totally blind, hydrocephalic, their hearts were very delicate, the blood vessels rudimentary and the yolk-sac was covered with dense networks of richly pigmented blood islands.

These monstrous embryos hatch only very rarely, most of them dying after the development has reached the stage in which the remant of the yolk-sac is in the normal embryo converted into the anterior body wall. As far as could be determined from the embryos in toto it is the enormously large (œdematous?) pericardia that mechanically obstruct the formation of the ventral body wall. The correctness of this interpretation will be tested by microscopic sections of these embryos.

The mechanism of the formation of the described monsters can at this time not even be definitely suggested. The observation was made that the yolk-sac in all extremely malformed embryos shows a marked decrease in size as compared with that of normal eggs of the corresponding stage of development. The greater the degree of injury inflicted on the embryo the smaller the yolk-sac. It is not impossible that the chemicals used in these experiments indirectly bring about this decrease in the size of the yolk-sac. For it was noticed that the chemicals used in these experiments softened the egg-membrane considerably, a fact which suggests an increase in the permeability of the egg. Owing to both increased permeability of the germ-disc cells and to internal osmotic pressure of the yolk-sac, an escape of substance from the yolk-sac might have been caused, which, being forced out at different points of the yolk-sac, might have fragmented the germ-disc. Many eggs were observed in which this fragmentation of the germ-disc was very evident. Some parts of this ruptured germ-disc may be so badly damaged as not to be able to develop further, while the remaining fragments, even if they are very small, may still give rise to various monsters, hemiembryos, dwarfs or even to a solitary eye. Or possibly the decrease in size of the yolk-sac of malformed ova may point to elimination of both yolk-sac and germ substance as an effect of the solvent action of the chemicals to which the eggs were exposed. Whatever the mechanism involved in the production of the recorded pathological ova may be, at the present time, it can hardly be more than conjectured. It will be the object of future investigations to find a satisfactory answer to this open question.

There seems to be a close similarity between these cases where parts of the germ-disc are apparently lost through elimination of some kind and the production of hemiembryos by mechanically injuring one of the blastomeres of the developing frog's egg, as described by Roux, Morgan and other investigators.

The writer intends to continue this work on the teleost eggs as well as on the amphibian and hen's eggs. He also hopes that he may in the near future secure adequate facilities for carrying on experiments on the influence of the toxic substances of pathological metabolism on the development of the mammalian embryo. The plan of this work would be to mate animals in which metabolic disturbances had been produced experimentally.

A complete description and analysis of the results obtained in the investigation reported here will be published at an early date.

The writer takes pleasure in acknowledging his indebtedness to Professor C. R. Stockard of Cornell University Medical College with whom he on several occasions had discussed some phases of the work, and from whom he has received valuable suggestions regarding preservation of material.

Marine Biological Laboratory,
Woods Hole, Mass.,
September 3, 1914.

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FEB 26 1915
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BIOLOGICAL BULLETIN

AN EARLY STAGE OF AN EXPERIMENTALLY PRODUCED EXTRAUTERINE PREGNANCY AND THE SPONTANEOUS PARTHENOGENESIS OF THE EGGS IN THE OVARY OF THE GUINEA PIG.[1]

Smithsonian Institution
FEB 26 1915
National

LEO LOEB.

The observation on which I wish to report is of great interest from several points of view. It explains the negative result of our former attempts which aimed at producing experimentally an extrauterine pregnancy in the guinea pig. It contributes to the understanding of the mechanism of the sexual cycle and it makes certain my previous conclusions, which formerly had only been probable, concerning the fargoing parthenogenetic development of ova in the ovary of the guinea pig, conclusions which our previous studies had made very probable. In a great number of previous experiments we made incisions in various parts of the uterus of the guinea pig and at different times after copulation.[2] Under these circumstances it certainly must often have happened that fertilized eggs left the uterine cavity. But extrauterine pregnancy did in no case take place under such circumstances. Even after ligation of the fallopian tubes we were not able to observe the occurrence of an extrauterine pregnancy. This latter observation is in accordance with some experiments of Mandl and Schmidt.[3] It was of interest to determine what was the fate of the ova which left the lumen of the uterus and passed into the peritoneal cavity after fertilization. An observation which we made in the course of our continued experiments serves to clear up this point.

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[1] From the pathological laboratory of the Barnard Free Skin and Cancer Hospital, St. Louis.

[2] Leo Loeb and John W. Hunter, *University of Pennsylvania Medical Bulletin*, Dec., 1908.

[3] *Archiv f. Gynaecol.*, 56, 1898.

Two days, sixteen hours after copulation, incisions were made into the uterus of a guinea pig. The weight of the animal at the time of the operation was 550 grams. The incisions were longitudinal and extended through both horns of the uterus up to near the point of juncture with the tubes. Besides the longitudinal incisions a number of transverse incisions into the uterine wall were made. Eighteen days after copulation uterus as well as one of the ovaries was taken out for examination. The ovary was cut into serial sections. Small follicles in the early stages of development, as well as other follicles in early stages of connective tissue atresia were found. In addition there were many follicles in the last stages of follicular atresia. There were also present several young corpora lutea, the center of which was partly filled out by connective tissue, while the center of the cavity had not yet been organized by connective tissue. There were furthermore present corpora lutea in an early stage of retrogression, as well as yellow bodies, completely atretic corpora lutea. These findings correspond to an ovary about three days after ovulation.

Microscopic examination of those parts of the uterus which had not been incised during the operation showed cylindrical surface and glandular epithelium with numerous mitoses in the glandular ducts. The fundi of the glands are somewhat smaller. In the lumen of the uterus there are some polynuclear leucocytes, a greater number of which are found in the ducts of the glands. In the connective tissue of the mucosa as well as in the surface epithelium the presence of several small round cells is noted. There are very few mitoses in the connective tissue of the mucosa which is rich in nuclei. These findings correspond to a condition of the uterus about 3–3½ days after copulation.

Near the tubal end of one of the uterine horns, not far from the usual situation of the ovary there was a small nodule. This nodule was cut in serial sections, and its structure is best explained by referring to the illustrations.

Fig. 1 shows the position of the embryo.

(*a*) It lies in the neighborhood of the fallopian tube.

(*b*) Some distance from the embryo we see the cut wall of the uterus.

(*c*) In the direction towards the tube we see the musculature of the uterus, in the opposite direction the epithelium with the glands and the connective tissue is visible. In the detached part of *c* the glands have the character of mucous glands. At this place the mucosa of the uterus is everted as a result of the incision. If we follow on further sections the position of the placenta which surrounds the embryo proper, we find that at some distance from the embryo proper it dips into the peritoneal side of the uterus at a place above the beginning of the incisions, where therefore the uterine lumen is still intact, and it even penetrates into a fissure of the musculature of the uterus. Further downwards the embryonal placenta extends to the peritoneal tissues of the upper part of the incised uterus. Fig. 1 of course represents only one section while the description which we just gave is based on a study of a number of serial sections. The egg embedded itself

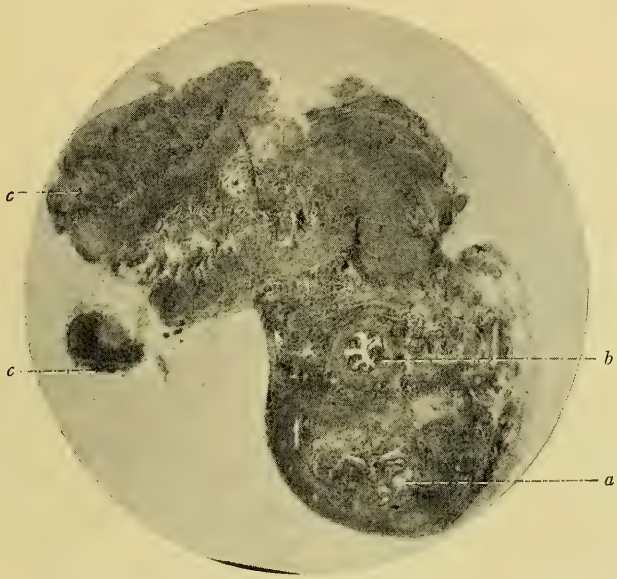

FIG. 1. Low power. *a*, embryo; *b*, Fallopian tubes; *c*, everted walls of the uterus.

A more detailed explanation of the figures is found in the text.

FIGS. 1, 2, 3, 4 and 6 are from microphotographs. Fig. 5 from a drawing.

evidently in the connective tissue between the upper end of the uterus and the lower end of the tube and its derivatives penetrated still deeper between the musculature of the uterus in the direction from the peritoneal side.

The character of the embryonal structures and their relation to the surrounding tissue are more clearly shown on Fig. 2. *b* is the fallopian tube. *a* is the embryo, which is surrounded by placental structures *c* and *d* and other similar not especially designated structures. In the periphery of these structures are found extensive hemorrhages into the connective tissues and these are in turn surrounded by strands of connective tissue and by blood vessels. The entire region between the tubes and the outer hemorrhagic zone is filled out by embryonal placenta.

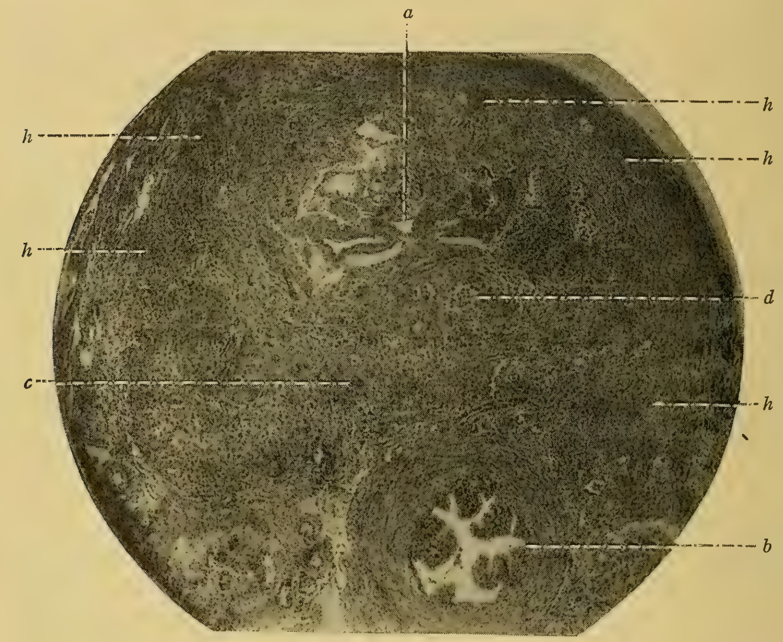

FIG. 2. The developing embryo; somewhat higher magnification. *a*, embryonal structure (neural canal?); *b*, Fallopian tube; *c*, giant cells of the embryonal placenta; *d*, cuboidal cells of the embryonal placenta surrounding cavities; *h*, hemorrhages in the surrounding connective tissue.

The embryo proper corresponds to a developing guinea pig at a stage directly following the formation of the germ layers.

A points to a central structure, which probably corresponds to the Anlage of the neural tube. Under the abnormal conditions under which the embryo must develop, the various embryonic structures are evidently somewhat distorted. Fig. 3 shows the central part of the embryo at a higher magnification. *A* points to the same cavity as *a* in Fig. 2. The cell designated by *a* is seen in the process of mitotic division. Other embryonal cells also divide mitotically at various places. Surrounding the central parts of the embryo we find epithelial structures arranged in layers adjoining as is shown on Fig. 2. Giant cells *c* surround the embryo at various places on Fig. 3 in a similar way as seen on Fig. 2. A larger number of giant cells are also found at a somewhat greater distance from the embryo. These giant cells are arranged typically around cavities, which are lined by smaller cuboidal cells. *D* on Fig. 2 points to such a cavity lined with such cuboidal cells. Perhaps also the canal *d* on Fig. 3 corresponds to such a cavity. The small cuboidal cells often proliferate and

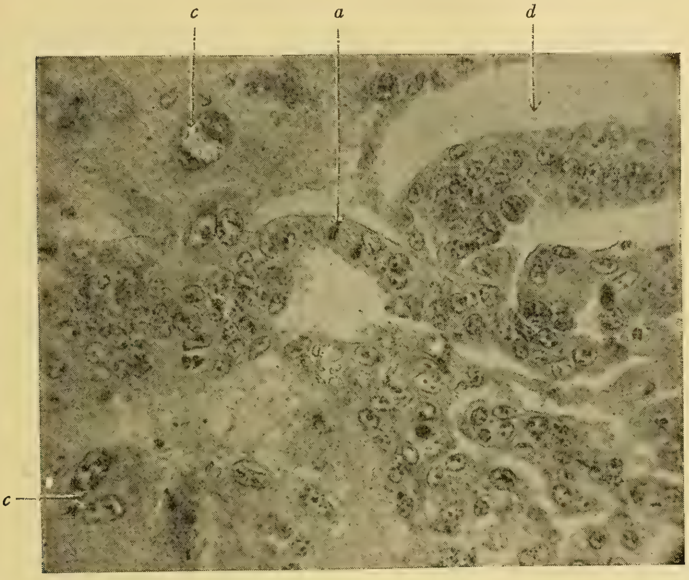

FIG. 3. The embryo proper, higher magnification. *a*, mitosis; *c*, surrounding giant cells; *d*, a structure which perhaps corresponds to the placental cavities lined with cuboidal cells.

their proliferation leads to the formation of papillary excrescences into the cavities. These excrescences fill sometimes a great part of these cavities. Mitoses often appear in these cuboidal cells. Fig. 4 shows such a placental structure at a higher magnification.

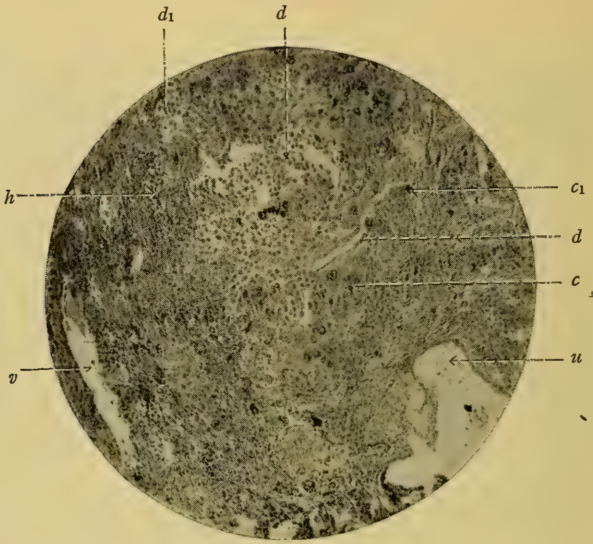

FIG. 4. A placental cavity lined with cuboidal cells. *c*, giant cells; *d*, cuboidal cells lining a cavity and forming papillary excrescences; *c′*, a giant cell penetrating into the surrounding connective tissue; *v*, blood vessels; *h*, hemorrhages in the connective tissue; *u*, experimentally misplaced uterine epithelium.

d points to a cavity lined with cuboidal cells. The cuboidal cells form papillary proliferations into the lumen. The cavity bulges into the surrounding tissue at *d*1. The cavity is surrounded on several sides by giant cells *c* and these giant cells protrude into the cavity and divide it into two parts. These giant cells have the power to penetrate farther into the surrounding tissue independently. *C*1 represents such a giant cell, which penetrates into the surrounding fibrous tissue. Surrounding this placental structure we find connective tissue in which there are many hemorrhages *h*. *U* represents a cavity lined with uterine epithelium. *v* represents a blood vessel. Fig. 5 represents a drawing of a similar placental structure. *D* represents the cavities lined with cuboidal cells, and partly filled with the proliferated cuboidal cells. *C* are the giant cells surrounding the cuboidal cells. *F* is fibrillary connective tissue in which there are many hemorrhages *h*. There is nowhere a formation of a decidua. *v* is a blood vessel.

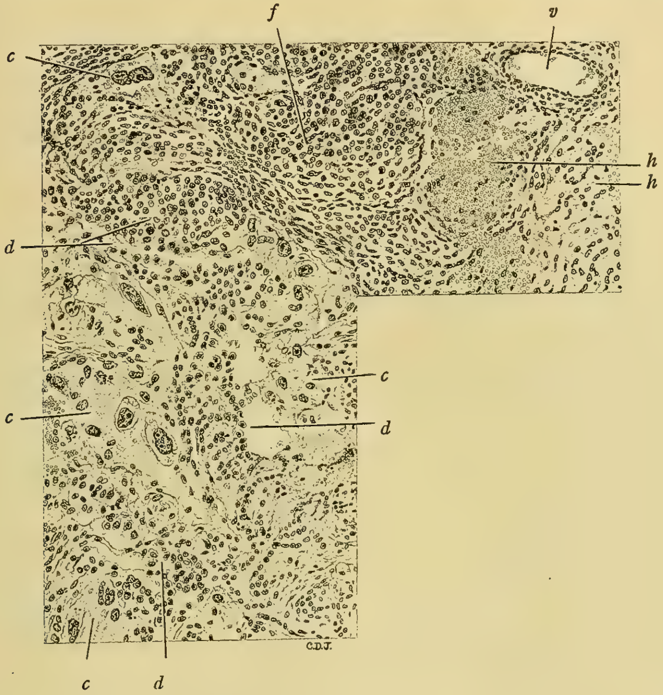

FIG. 5. A typical placental structure. The various letters have the same significance as in Fig. 4.

As we have already seen on Fig. 4, the giant cells penetrate deeper into the tissue, independently of the small cuboidal cells. They prefer especially the neighborhood of blood vessels, penetrate the walls of the latter and replace the endothelial cells. Blood vessels thus changed are of course thereby weakened, and they are no longer as well able to resist to the full extent the blood pressure, and thus hemorrhages into the tissue, as so frequently seen, result. Fig. 6 shows two vessels *v*. Giant cells *c* have advanced up to the lumen of these vessels and substitute the endothelial cells. In *c*1 also there lies a giant cell in the tissue. At many places there are hemorrhages *h* in the connective tissue. In the periphery of the upper half of the section, connective tissue surrounds the structure. *d* points to a cavity filled with small cuboidal cells.

These findings will have to be interpreted in the following way. At the time when the incisions were made into the uterus, namely two days and sixteen hours after copulation, the ova had probably already left the tube and had reached the upper part of the uterine cavity. At this time one or more of the ova left the uterine cavity through the incision into the uterine wall and one of the ova passed around the outer side of the upper end of the left uterine horn, and embedded itself in the connective tissue between the tube and the upper end of the left uterine horn. A part of the embryonal placenta in the course of development penetrated farther into the musculature of the uterine horn. The fertilization of this ovum had in accordance with the

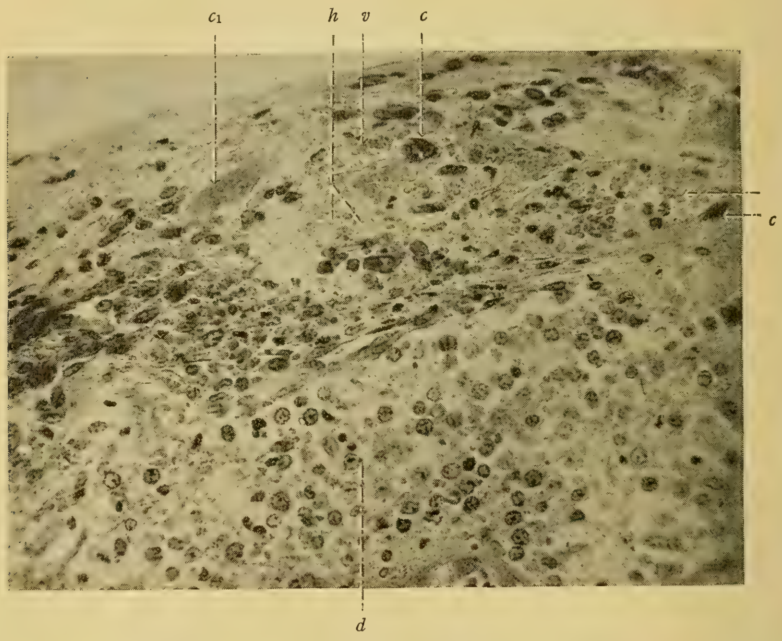

FIG. 6. Placental embryonal giant cells penetrate into the vessel wall. *v*, bloodvessels; *c*, giant cells substitute vascular endothelial cells; *c'*, a giant cell lying in the host tissue; *h*, hemorrhages in the host connective tissue; *d*, cuboidal placental cells of embryonal origin.

general view concerning the time of fertilization of the guinea-pig ovum already taken place at the time of the operation. We excised the nodule fifteen days eight hours after the incisions had been made.

Our description of the embryo clearly shows that under the existing abnormal conditions the development of the ovum was greatly retarded. The embryo is still alive and even growing, as the mitoses, which were found at various places, indicate, but the embryo is found to be at a very much earlier stage of development than one would expect eighteen days after copulation. The embryonal placenta also is only very incompletely developed. While the normal placenta of the guinea pig shows a complicated structure at this period of development, in our case the embryonal placenta consist solely of layers of small cuboidal cells, which usually line cavities, and produce papillary excrescences projecting into the cavities. On the outer side of these cavities there are giant cells. The giant cells penetrate also independently into the surrounding connective tissue and substitute walls of blood vessels, and thus contribute to the hemorrhages which we find so frequently. Cuboidal cells as well as giant cells are growing actively by mitosis—the latter however to a lesser degree. The surrounding host tissue remains passive. The embryonal tissue is surrounded by fibrillar connective tissue containing the ordinary connective tissue cells. *There is nowhere an attempt at the formation of a decidua on the part of the host tissue.*

These observations are in entire accord with our former experimental findings from which we concluded that in the guinea pig solely the connective tissue of the uterine mucosa is able to produce decidua in response to artificial stimuli, as cuts and foreign bodies while the fallopian tube, peritoneal and other connective tissue are unable to do so.[1]

These additional observations again prove the similarity in the mode of action of the artificial stimuli leading to the formation of a decidua on the one hand and of the ovum on the other hand. In a similar manner as the artificial stimuli were not able to call forth a

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[1] Leo Loeb, *Zentralblatt für Physiol.*, Bd. XXIII., No. 3; *Journal Am. Med. Association*, Vol. LIII., p. 1471, 1909.

formation of decidua in the peritoneal connective tissue, the ovum is likewise unable to do so.[1]

These observations furthermore clear up the fate of the ovum in cases in which it is not able to develop normally in the uterine wall. Frequently a fixation of the ovum does not take place in such abnormal cases, especially on the smooth peritoneal epithelium. In other cases however the ovum fixes itself and begins to develop in the connective tissue without however finding the necessary decidual reaction on the part of the surrounding connective tissue. In such cases the development of the embryo proper as well as of the embryonal placenta is very much retarded as compared to the normal development; furthermore the embryonal differentiation also remains incomplete and we may assume, that after some time the growth ceases and the embryonal structure is substituted by host connective tissue in a similar manner as in the ovary of the guinea pig. We find therefore in the guinea pig no or only a very much retarded and incomplete development of the ovum outside of the uterus. This is in all probability due to the fact that the host tissue is not suited to receive the ovum and to supply it with the necessary food stuffs. In this case the host tissue behaves passively in contradistinction to the uterine mucosa. This conclusion agrees with the fact that we find a general parallelism in the ability of the uterine mucosa to produce decidua or deciduomata and to permit a normal development of the ovum. As I have previously shown, various experimental interferences, as for instance extirpation of the corpora lutea or of the ovaries, have approximately to the same extent an inhibiting influence on the development of deciduomata and of pregnancy. We may thus conclude that the ability of the host connective tissue to produce a decidua in a normal manner is of significance for the normal development of pregnancy.

We see therefore that in the guinea pig the ovum does either not develop at all outside of the uterus or in case an extrauterine fixation of the ovum should take place, the development is much retarded and soon comes to a standstill. As our present and especially our previous observations concerning the parthenogenetic pregnancy in the ovary of the guinea pig demonstrate,[1] the development of the embryonal placenta preponderates relatively very much over that of the embryo proper, probably because as I have already suggested, in contact with the host tissue the derivatives of the ovum produce mainly the placental structures. This is very marked in the case of the parthenogenetic development in the ovary of the guinea pig, where in typical cases under those conditions placental structures are found exclusively and only exceptionally the embryo proper begins to develop.

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[1] As we shall later especially emphasize, the same holds good in the case of the parthenogenetic development of the egg in the ovary of the guinea pig.

These observations explain apparently very well the fact, that while in the guinea pig a further going development of the ovum is possible *after extrauterine fixation,* in man a complete extrauterine development is not an infrequent occurrence. Our findings suggest as one of the causes for this difference in occurrence of extrauterine pregnancy in man and guinea pig, the fact that in the case of man the host tissue offers a more suitable soil than in the case of guinea pig; while as we saw in the latter the development of the decidua in response to various kinds of stimuli takes only place in the connective tissue of the uterine mucosa, in the case of man the connective tissue of various pelvic organs and even the appendix is able to produce decidua as many observations show. In accordance with this interpretation a number of observers actually reported the development of a decidua in the fallopian tube in cases of tubal pregnancy. It is very probable that in cases of tubal pregnancy in which a decidua was not found in the tube, we had to deal with stages in which the chorionic wandercells had already penetrated deep into the host tissue and thus gradually destroyed the decidua; in a similar manner in the case of the guinea pig it can readily be seen that the wandercells of the embryonal placenta destroy a greater part of the decidua. It is very probable that from a certain stage of embryonal development on, the decidua is no longer indispensable as far as the continued existence and further development of pregnancy are concerned.

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[1] *Roux's Archiv,* Bd. XXXII., p. 662, 1911; *Zeitschrift f. Krebsforschung,* 11. Bd., 2. Heft, 1912.

Our observations are also of interest from another point of view. We know that under ordinary circumstances the corpus luteum remains longer preserved in pregnancy than in the non pregnant animal. Pregnancy prolongs the sexual cycle. We may now inquire into the cause of the prolongation of the life of the corpus luteum during pregnancy. Several years ago I pointed out, that the growth of the embryo might perhaps directly or indirectly prolong the life of the corpus luteum during pregnancy.[1]

Now we find in our case a small embryo as well as an embryonal placenta developing outside the uterus. Notwithstanding this fact a new ovulation had taken place about three days previously and accordingly the corpora lutea of the preceding sexual cycle which had been terminated at the time of the last ovulation were degenerated.

This observation proves that a developing embryo including embryonal placenta is in itself not sufficient to protect the corpus luteum from degeneration, and to prevent a new ovulation. It is possible that the maternal placenta is concerned in the prolongation of the life of the corpus luteum either alone or in connection with the embryo, which latter as our further experiments have shown, prolongs noticeably the life of the experimental placentomata (deciduomata). Indeed experiments which I carried out some time ago have shown that the development of deciduomata without the development of an embryo is able to prolong the sexual period; while normally the sexual cycle in the guinea pig has a duration of from 15 to 18 days, it lasts from 20–30 days after production of deciduomata.[2] Whether as a result of these experimental interferences also the life of the corpus luteum is prolonged will have to be still further investigated.

Our observations are furthermore of significance for the interpretation of certain structures, which I found in about 5 per cent. of the ovaries of young guinea pigs.[3]

In as much as these structures become absorbed after a certain time and are substituted by connective tissue, these structures

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[1] *Zentralblatt f. Physiol.*, Bd. XXIV., Nr. 6; *Medical Record*, June 25, 1910.

[2] Leo Loeb, Biological Bulletin, Vol. XXVII., July, 1914.

[3] *Arch. f. mikrosk. Anatomie*, Bd. 65, 1905; *Roux's Archiv*, Bd. XXXII., p. 662, 1911; *Zeitschrift f. Krebsforschung*, 11. Band; 2. Heft, 1912.

must in fact occur more frequently than the direct findings suggest. We have to deal with formations which resemble closely structures of the embryonal placenta, and they originate in ovarian follicles. They are either well preserved or are found in the process of retrogression and in the end are substituted by connective tissue. In two cases I was able to find besides embryonal structures proper, for instance the Anlage of the nervous system. It had been known previously and I myself had described processes which had to be interpreted as the first segmentations of eggs in atretic follicles which in consequence of the abnormal conditions under which they took place followed as might have been expected an abnormal course.[1] The interpretation that we have to deal merely with the disintegration of the ova can be excluded with certainty. Such an interpretation would be contradicted by the regularity of the divisions. Furthermore we may find in these various segments either nuclei or the remnants of nuclear spindles and I was able to observe the simultaneous presence of a mitosis in each one of the two such segments. These segmentations also are found chiefly in the ovaries of the young guinea pigs. A somewhat furthergoing formation of the first segments in ovarian eggs has recently been described in armadillo by Newman.[2]

In all these cases we have merely to deal with the first parthenogenetic segmentations of the ovum, while our observations in the ovary of the guinea pig prove a much furthergoing development leading to the formation of embryonal placenta and of embryos in the stage of the germ layers within the ovary. It is of course natural, as I emphasized on a former occasion, that under these abnormal conditions the processes of development cannot follow an altogether normal course, and it was therefore desirable that a confirmation of our interpretation of these ovarian structures should be obtained. The findings which we have just communicated offer the desired confirmation. In our new observations we have also to deal with embryonal structures found in the peritoneal connective tissue and developing in an abnormal situation without being aided by the host tissue through the formation of a decidua. We have of course to consider the fact that in the ovary the limitation of space is still more marked than in the connective tissue on the outer side of the fallopian tube and of the uterus. In both cases the placental structures preponderate over the embryonal ones proper; in both a retardation in the development is found and a preponderance of certain placental structures. Such favored structures are the layers of cuboidal cells, lining cavities, forming papillary excrescences into these cavities and surrounded at the periphery by giant cells which latter penetrate in both cases into the surrounding tissue, especially around the blood vessels, the walls of which they may perforate, thus giving rise to hemorrhages. The identity of both formations, namely of the experimentally produced extrauterine pregnancy which we have just described and of the embryonal structures developing parthenogenetically in the ovary becomes quite evident, when one compares the microscopic sections of both of these formations. The microphotographs and the drawings also show the similarity.

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[1] Leo Loeb, "On Progressive Changes in the Ova in Mammalian Ovaries, *Journal of Medical Research*, Vol. VI., 1901. *Arch. f. mikrosk. Anat.*, Bd. 65, 1905.

[2] H. H. Newman, BIOLOGICAL BULLETIN, XXV., p. 52, 1913. It may be especially emphasized that our interpretation of the placental and embryonal structures found by us in the ovaries of guinea pigs does in no way depend on the interpretation of those changes in the ova within the ovaries of the guinea pig which in common with previous authors we held to be early abnormal segmentations of ova, while a number of other investigators interpreted them as of a degenerative character. There can be no doubt about the presence of further developed embryonal structures in the ovaries of guinea pigs.

The similarity of the embryonal structures proper becomes clear through a comparison of Figs. 2, 3, and 6 in the former communication (*Zeitschrift für Krebsforschung*),[1] and of Figs. 2 and 3 in the present communication. The similarity of the placental structures is made evident through a comparison of drawings 1, 2 and 4 in the *Archiv f. mikrosk. Anatomie*,[2] of the Figs. 10, 12, 14 and 15 in the *Zeitschrift für Krebsforschung* with Figs. 4 and 5 of the present article. On several of these former figures there were also represented the relations of the wandering giant cells to the blood vessels and the hemorrhages resulting therefrom.

Our new observations render it therefore certain that a fargoing parthenogenetic development of ova takes place in the ovaries of a

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[1] *Loc. cit.*

[2] *Loc. cit.*

relatively large number of guinea pigs, leading in the first place to the formation of placental structures, in some cases however also to the formation of embryos in the stage of the germ layers. We have discussed the possible causes for this parthenogenetic development on another occasion.[1] We have perhaps to deal with a development which is caused by changes in the circulation and in the exchange of gases at the time and in consequence of the rupture of follicles.

Such an explanation would be in accordance with the fact that the first segmentations of the ovum in the ovary of the guinea pig are found especially in atretic follicles, that the segmentations set in with beginning atresia and then gradually progress. Now we know that the atresia of follicles is more marked, than at any other time, at the time of ovulation.[2] In this connection it is especially worthy of notice that the first segmentations of the ova in the ovary as well as the furthergoing parthenogenetic development, which leads to the formation of embryonal and placental structures, is preferably found in the ovaries of young animals. The latter, however, occurs occasionally also in somewhat older guinea pigs. In such cases we may perhaps have to deal with structures which originally developed in younger animals, which then however had remained stationary for a longer period of time.

We have still to discuss the significance of these structures for the interpretation of certain pathological formations, namely the embryomata and the chorion epitheliomata of the female germ gland. The large majority of pathologists assume in agreement with the suggestion of Bonnet and Marchand that these pathological structures take their origin from misplaced blastomeres and not from the parthenogenetically developing ovum. As I formerly emphasized[3] our observations make it very probable that such pathological formations originate from parthenogenetically developing ova. They are therefore the "descendants" and not the "brothers" of the organism in which they originate. We may assume that in certain cases the parthenogenetic develop-

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[1] Leo Loeb, *Proceedings Am. Philosophical Society*, Vol. L., p. 228, 1911.

[2] Leo Loeb, *Journal of Morphology.*

[3] *Zeitschrift f. Krebsforschung, loc. cit.*

ment of ova leading to these pathological structures begins only after birth. In a similar manner as we saw that embryonal placenta as well as the embryo proper can develop from the parthenogenetically segmenting ovum and that the embryonal placenta can be formed without the simultaneous development of the embryo proper, thus chorion epitheliomata may originate in the ovary without any accompanying embryonal structures proper. In other cases however there develop mainly the embryonal structures proper or certain of their parts.

This conception of these structures explains the fact that they are mainly found in the germ glands. On the other hand, there exists no reason, why we should expect that aberrant blastomeres should mainly be found and develop at this place. Furthermore I have never been able in the many hundreds of ovaries of guinea pigs which I have examined microscopically to find a structure resembling a misplaced blastomere.

We still have to explain why these structures are occasionally also found in the male germ glands and especially, why teratomata occur also, although less frequently, at other parts of the body, outside of the germ glands. As far as their occurrence in male germ glands is concerned, it might be explained by the fact that in a certain number of cases cells of both sexes may be found in the same individual, that therefore true hermaphroditism occurs. That this is not so rare an occurrence as has been assumed has recently been shown by L. Pick.[1] We have perhaps also to consider the possibility that at a certain stage of development also the male germ glands are capable of developing in a similar manner as the ova. However at the present time there exist no facts supporting such an hypothesis.

We know furthermore that in the course of embryonal development the germ cells migrate. It is therefore conceivable that occasionally one of their number may follow a wrong path and thus give origin to the formation of the teratomata outside of germ glands. While we are thus able to explain the origin of these structures on the basis of a parthenogenetic development of ova we do not intend to deny the possibility that under certain conditions irregularities in the embryonal development may lead to the transformation of blastomeres or of remnants of not fully differentiated embryonal tissues into teratomata, an hypothesis which would be in accordance with the finding of misplaced blastomeres by W. Roux in the course of the embryonal development of amphibian eggs.

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[1] Cited from a review in the *Münch. med. Wochenschrift*, 1913.

SUMMARY.

1. It is possible in the case of guinea pigs to produce experimentally the first stages of an extrauterine pregnancy.

2. In a similar manner, as in the case of guinea pigs experimental interferences of various kinds are not able to call forth the production of deciduomata in the connective tissue outside of the uterine mucosa after the discharge into the circulation of the sensitizing substance which is secreted by the corpus luteum, the developing ovum is unable to call forth a decidual reaction.

3. Under the conditions produced by us experimentally the development of the embryo is very much retarded and will in all probability come to a standstill after some time. Neither does the embryonal placenta develop in an entirely normal manner, although quantitatively the embryonal placental structures preponderate considerably over the embryonal proper. It is very probable that the lack of the decidual and of the typical blood vessel reaction on the part of the host connective tissue is the cause of this abnormal development. In man an extrauterine decidua can develop and accordingly here a fully developed extrauterine pregnancy is not rare. As we have shown previously the effect of the extirpation of the corpora lutea on the formation of the decidua and on the development of pregnancy is approximately parallel. This is an additional fact which renders probable the significance of the decidual reaction for the complete development of the extrauterine pregnancy. The decidual reaction is at least one of the conditions which has to be considered in this connection.

4. Notwithstanding the presence of a young, developing embryo in the extrauterine connective tissue a degeneration of the corpora lutea and a new ovulation took place in the ovary. This proves that the persistence of the corpora lutea during pregnancy does not depend upon a substance secreted by the embryo; it is

probable that the growth of the decidua perhaps in combination with the growth of the embryo prolongs directly or indirectly the life of the corpora lutea during pregnancy. Thus far experiments, which we have carried out in order to decide this question, have shown that the presence of living and growing deciduomata prolongs the sexual period; furthermore that pregnancy prolongs the life of the deciduomata. Further investigation will decide whether or not these effects are exerted indirectly by means of the corpus luteum.

5. Our experiments render it certain that the structures which we found in a considerable number of guinea pigs and which we formerly interpreted as early stages of parthenogenetically developed pregnancies in the ovaries of guinea pigs really represent a relatively far going parthenogenetic development of ova which may lead to the formation of embryos in the germ layer stage which however usually leads merely to the formation of an embryonal placenta probably as a response of the developing ovum to the influence exerted by the contact with the surrounding host tissue. We show furthermore the significance these findings have for the interpretation of the teratomata and chorionepitheliomata of the germinal glands.

6. The embryonal wander cells destroy outside as well as within the wall of the uterus bloodvessels of the surrounding host tissue in the ovary as well as in the peritoneal connective tissue and they thus cause hemorrhages in the surrounding host tissue.

SOME CRYOSCOPIC AND OSMOTIC DATA.[1]

WALTER E. GARREY.

Subsequent to the publication in 1905 of data on "The Osmotic Pressure of Sea Water and the Blood of Marine Animals, etc.," (1) the author has had occasion in the course of his other investigations, to make numerous determinations of the freezing point of various sea waters, solutions and bloods; this method having been used to check up other methods of obtaining solutions of known osmotic pressures. Some of the data thus acquired have been correlated, and although somewhat fragmentary, they are published in hopes that they may facilitate the work of other biologists.

The determinations have been made with the Beckmann apparatus and a differential thermometer, which could be read accurately to 0.005° C. When it is remembered that the depression of the freezing point (Δ) of a gram-molecular solution of a non-electrolyte is (theoretically at least) 1.85° C. below zero, that this depression corresponds with an osmotic pressure of 22.4 atmospheres (at 0° C.), and that the osmotic pressures vary directly with the depression of the freezing point, it is seen that the osmotic pressure of any solution may be calculated from the simple formula: osmotic pressure $= 22.4$ a. $\Delta/1.85$.

SEA WATERS.

Sea waters are not solutions of absolutely fixed chemical composition, nor have they a constant concentration. While the ratios of certain salts are quite constant, there are other variations such as the content of absorbed oxygen and carbon dioxide and even of the fixed carbonates. J. Loeb (2) has called attention to the fact that the free alkalinity, *i. e.*, the number of HO ions, is distinctly higher in the sea water at Woods Hole than at Pacific Grove.

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[1] From the Physiological Laboratory of Washington University, St. Louis.

The figures for the depression of the freezing point (Δ) given in Table I. indicate the wide range in concentrations in sea waters of different localities.

TABLE I.

Sea Water from:	Δ — °C.	Observer.	Reference.
Naples	−2.29	Bottazzi	*Arch. ital. de biol.*, 1897, XXVIII., 61.
Arcachon	−1.89	Rodier	Trav. des Lab. d'Archachon, 1899.
Pacific Grove, Cal.	−1.925	Greene	Bull. U. S. Bureau Fisheries, 1904. XXIV., 429.
Pacific Grove, Cal.	−1.90	Garrey	BIOL. BULL., 1905, VIII., 257.
Woods Hole	−1.81	"	BIOL. BULL., 1905, VIII., 257.
Beaufort, N. C.	−2.04	"	1911.
Helgoland	−1.90	Dakin	Bio-Chem. Jour., 1908, 269.
In the Kattegat	−1.66	"	
Open Baltic Sea	−1.30	"	
Kiel harbor	−1.093	"	

In the following sections further details obtained by the author, by means of the cryoscopic method, are given for sea waters of some American localities.

(*a*) *Woods Hole.*—Determinations made during the summer of 1904 have been previously reported (*loc. cit.*, pp. 258–259) showing the freezing point to be slightly variable between −1.805 and −1.84° C. The average of determinations made the latter part of July of six different years gave an average Δ = − 1.81° C,. with which, as will be seen from succeeding data, the following solutions are isosmotic: Sodium chloride, 0.52 *m*; Magnesium chloride, 0.29 *m.*, cane sugar, 0.73 *m.* "Van't Hoff's solution," made from *m*/2 stock solutions, had a freezing point of − 1.84° C., this is so slightly in excess of the concentration of Woods Hole sea water that it may be considered isosmotic with it. This "Van't Hoff's solution" was made up from half molecular solutions according to the formula given by J. Loeb, (3) viz: 100 molecules NaCl, 2.2 molecules KCl, 1.5 molecules $CaCl_2$, 7.8 molecules $MgCl_2$ and 3.8 molecules $MgSO_4$. The traces of bicarbonate and phosphate were omitted from the solution, but when added in optimum amounts (*e. g.*, 1 c.c. N/20 $NaHCO_3$ per 100 c.c. solution, as in the procedure of Loeb, p. 35), the solution becomes exactly isosmotic with Woods Hole sea water.

(*b*) *Pacific Grove.*—Green in 1904 made freezing point determinations of the Pacific Grove sea water and found that Δ = − 1.924° C. Garrey in 1905 made determinations, obtaining a slightly lower value for Δ viz., − 1.905° C. On the basis of either of these figures, it is seen that the sea water in this locality is about 5 per cent. more concentrated than at Woods Hole and that a correction for this amount must be made if the osmotically equivalent solutions are to be calculated from the figures given in the previous section (*a*).

(*c*) *Beaufort, N. C.*—Working in the laboratories of the U. S. Bureau of Fisheries during the summer of 1911, the author made the following observations of the freezing point of sea water obtained at different localities in that vicinity and under different conditions as described in Table II.

TABLE II.

1911.	Beaufort, N. C.	Δ = °C.	Remarks.
June 8	Open sea, outside "Sea Buoy"	−2.043	
" "	Wharf of U.S.F.C.	−1.987	11:40 a.m.—tide low, N.E wind, previous showers.
	Wharf of U.S.F.C.	−2.015	4:00 p.m. tide high
July 19	Wharf of U.S.F.C.	−2.038	
" "	Open sea, at "Sea Buoy"	−2.03	
" 20	Wharf, U.S.F.C.	−2.07	9:15 a.m., tide low
" 25	Wharf, U.S.F.C.	−2.06	3:00 p.m., tide high
" "	Bogue Sound	−2.073	
" "	Wharf U.S.F.C.	−2.079	Tide low, strong south wind
Sept. 6	Sea Buoy	−2.05	
" 8	Wharf, U.S.F.C.	−2.052	Tide low
" "	Newport River, at "Cross Rocks"	−1.707	

From these figures it is seen that the open sea water off Beaufort has a Δ = − 2.04° C., and is 12 per cent. more concentrated than at Woods Hole.

(*d*) *Diluted Sea Water.*—The constant necessity for the use, in biological investigations on marine forms, of diluted sea water and corresponding concentrations of pure salts, has led the author to make the determinations found in Table III. Various dilutions of Woods Hole sea water were made and the freezing points determined. In most cases the densities at these dilutions have also been determined by the pycnometer method. The figures given in the table are all from actual determinations made by the author; when not given they may be approximated by interpolation.[1]

TABLE III.

Dilution: Woods Hole Sea Water c. cm. +	Distilled Water c. cm.	$\Delta = {}^\circ C.$	Densities of Sea Water Dilutions at 21.5° C. (Ref. H_2O at 21.5° C.)	NaCl with same Δ, Gms. in 100 cc. of Solution.
Undiluted	0	−1.81	1.02426	3.04
85 c.c.	15 c.c.	−1.54		2.6
75 "	25 "	−1.35		2.275
66⅔ "	33⅓ "	−1.20		2.00+
60 "	40 "	−1.09		1.81
50 "	50 "	−0.915	1.0123	1.58
45 "	55 "	−0.82		1.4
40 "	60 "	−0.73	1.0096	1.21
35 "	65 "	−0.64		1.07
33⅓ "	66⅔ "	−0.61	1.008	1.02
32 "	68 "	−0.595		1.00
30 "	70 "	−0.547	1.0073	0.91
25 "	75 "	−0.460	1.0062	0.76
20 "	80 "	−0.37	1.0046	0.60
10 "	90 "	−0.187	1.0023	0.30

CANE SUGAR.

Attention should be directed to a fact to which Jones (4), Morse and Fraser and Berkeley and Hartley have called attention, viz., that cane sugar solutions show osmotic pressures considerably in excess of what theory would lead one to expect. Loeb has shown the importance of this fact for biological work (5). From purely theoretical considerations one would expect a molar (gram-molecular) solution to show an osmotic pressure only slightly in excess of that of Woods Hole sea water. Loeb found that it caused a shrinkage of the eggs of the echinoderms even of the Pacific, and his experiments caused him to select 6/8 m. cane sugar as the proper concentration for the development of Strongylocentrotus purpuratus. The osmotic pressure of Woods Hole sea water by calculation from the freezing point is 21.9 a. (at 0° C.), a figure which is almost identical with that obtained by calculation for 0.75 gram molecular solutions of cane sugar, using the measurements of Berkeley and Hartley.[1] Some of our determinations of the freezing point of solutions of cane sugar illustrate their peculiar osmotic behavior.[2]

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[1] The determinations of Gerlach for NaCl and KCl (*Chemiker-Kalender*, 1914, I., p. 261) and of Schiff for MgCl and $CaCl_2$ (*ibid.*, p. 265) show, that, for concentrations of solutions of the magnitudes with which we are dealing and in which these salts are present in sea water, the densities are a linear function of the concentration. A plat of our determinations shows the same to be true for both densities and freezing points of dilutions of sea water.

For a gram molecular solution of cane sugar (342.2 grams per liter *of solution*) we found $\Delta = -2.775°$ C.; for ¾ mol. (256.6 grams per liter) $\Delta = -1.855°$, $-1.86°$ C.; and for ½ mol. (171.1 grams per liter) $\Delta = -1.15°$, $-1.155°$ C. For these three solutions the theoretical depression of the freezing point would be to $-1.85°$, $-1.387°$ and $-0.925°$ C., respectively. Comparison of these figures shows how much in excess of the theoretical osmotic pressure, that of these solutions really is. Morse and Fraser have pointed out that the correspondence with the theoretical expectations is greater, if "weight normal" solutions are used, *i. e.*, if the substance is present in a liter of the solvent, instead of this volume of the solution. This does not account, however, for the full amount of the discrepancy found. To illustrate this: It was found in our experiments that in making a gram-molecular solution by dissolving 17.11 grams of cane sugar in 50 c.c. of the solution (15° C.), it was necessary to add only 39.4 c.c. of distilled water; Δ was $-2.775°$ C. Had 50 c.c. of solvent been used to make the corresponding "weight normal" solution, Δ would have been $-2.187°$ C.[3] This figure exceeds the theoretical Δ ($-1.85°$ C.) by 0.337° C., which is probably to be accounted for by hydration of the sucrose molecule (Callendar (6)).

In the figures given above it is to be noted that the Δ of .75 mol. solution of sucrose ($-1.855°$ C.) is that which theory expects of a gram molecular solution, and its osmotic pressure lies between that of the sea water at Woods Hole and Pacific Grove. By extrapolation we obtain the following figures for the concentration of sucrose; isosmotic with sea water of:

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[1] The measurements of Berkeley and Hartley were made using other concentrations. The original figures of these workers as also those of Morse and Fraser et al. are given in the " Physikalisch-chemische Tabellen," Landolt, Börnstein and Roth, 4th ed., Table 179, p. 787. Their original papers are referred to, *ibid.*, p. 790.

[2] The sugar used in these experiments was free of all reducing sugars and had been twice recrystalized from glass-distilled water with subsequent drying in vacuo.

[3] Calculation of the freezing point of a molecular "weight normal" solution, based upon Morse's figure for the observed osmotic pressure (24.8 a. —0° C.) gave a slightly lower figure, viz.: $\Delta = -2.048°$ C.

1. Woods Hole = 0.73 m. (Δ = − 1.81° C.)
2. Pacific Grove = 0.765 m. (Δ = − 1.90° C.)
3. Beaufort = 0.81 m. (+) (Δ = − 2.01° C.)

SALT SOLUTIONS.

(*a*) *Sodium Chloride.*—In addition to the freezing points of solutions given in Table III., the following have been determined by the author on solutions made up by different competent workers. The concentrations chosen were somewhat

TABLE IV.

NaCl, Molecular Concentration.	Made by	Δ = ° C.	Remarks.
0.65	L.	−2.255	
0.65	G. 13° C.	−2.35	
0.65	G. 22° C.	−2.50	Made in a flask standardized to 15° C.
0.60	T.	−2.11	
0.58	G.	−2.03	Isosmotic with Beaufort Sea water
0.54	G.	−1.90	Isosmotic with Pacific Grove Sea water
0.54	L.	−1.895	
0.52	G.	−1.81	Isosmotic with Woods Hole sea water.
0.50	L.	−1.735	
0.50	L.	−1.74−	
0.50	G.	−1.745	
0.50	F.	−1.75	
0.50	L.	−1.765	
0.50	G.	−1.745	
0.444	G.	−1.54	

TABLE V.

$MgCl_2$ Concentration.[1]	Δ = ° C.	Remarks.
0.50 molecular	−2.845	
0.36 "	−2.03	Isosmotic with sea water at Beaufort.
0.35 "	−1.985	
0.31 "	−1.895	Isosmotic with sea water at Pacific Grove (or 0.32 m. according to Greene's determination).
0.30 "	−1.85	
0.29 "	−1.815	Isosmotic with sea water at Woods Hole.
0.10 "	−0.495	Dissolved in 100 c.c. of distilled water (not of solution).

[1] Concentration referred to volume of solution, not of solvent.

to either side of those isosmotic with the sea waters of our coast laboratories.

(*b*) *Magnesium Chloride.*—In solutions of this salt some of the molecules are dissociated into three ions, which accounts for the fact that the osmotic pressure is greater and consequently the depression of the freezing point is lower, than that of equimolecular solutions of sodium chloride. The following commonly employed solutions have been tested (cf. Table V.).

ANIMALS.

In addition to data previously published by the author (*loc. cit.*, p. 263), several determinations have been made on the blood of animals of the waters of the American coast, and inland rivers.

(*a*) *Limulus polyphemus.*—In the work referred to it was shown that under experimental conditions the blood of this animal, like that of other marine invertebrates varies to conform in concentration to that of the external medium. We have since found that this is true in the natural habitat of these animals, thus at Woods Hole (1904) the blood of Limuli depressed the freezing point like sea water, to – 1.82° C. At Beaufort, N. C., July 20, 1911, the water of the Fisheries "pound" froze at – 2.03° C. The blood of four Limuli taken from this water, in which they had been kept for several weeks, showed the following freezing points, respectively, – 2.025°, – 2.03°, – 2.04° and – 2.35° C. In the case of another *Limulus* captured at "Cross Rocks" in the Newport River, near Beaufort, September 8, 1911, the blood $\Delta = -1.71°$ C. while the water at that place depressed the freezing point to – 1.707° C. Such readings taken from animals under natural conditions established the absolute identity of osmotic pressure of the external and internal media despite the differences in their composition.

(*b*) *Elasmobranchs.*—A shark seven feet in length (not identified) was captured in the Fish Commission nets at Beaufort, July 27, 1911; the sea water froze at – 2.02° C.; cryoscopic readings of the blood from the heart and portal vein, withdrawn immediately after death, were identical, within 0.01° C., and showed Δ to be – 2.182° C., which again is practically identical with that of the Beaufort sea water taken in the neighborhood of the fish trap on that date.

The Δ for this elasmobranch is larger than for those tested at Woods Hole by the author and later by Scott (7). The figures indicate an adjustment to the greater concentration of the sea water at Beaufort, a fact which is also borne out by determinations made on the blood of "sting rays," the blood of four of which at Beaufort gave $\Delta = -1.98^\circ, -2.04^\circ, -2.03^\circ, -2.07^\circ$ C., respectively. These depressions are not greater than that of the sea water from which the animals were taken although both the author and Scott found a slightly greater depression for the blood of the dog fish (*Mustelus canis*) of Woods Hole than for the sea water of the laboratories; this water is, however, somewhat less concentrated than the water outside the heads.

(*c*) *Marine turtles.*—The defibrinated blood of three species of marine turtles[1] caught at Beaufort in 1911 was frozen and the Δ thus determined for each individual is as follows:

Chelonia mydas $\Delta = -0.675^\circ$ C.
Colpochelys kempi $\Delta = -0.687^\circ, -0.70^\circ, -0.70^\circ$ C.
Caretta caretta $\Delta = -0.69^\circ, -0.69^\circ, -0.685^\circ$ C.

In the cases of two carettas obtained at Woods Hole in 1913, the Δ found was identical with that given above. These depressions (Δ) are, in all cases, greater than those obtained by Bottazzi for "Thalassochelys caretta" ($\Delta = -0.61^\circ$) although it is worthy of mention that the waters from which our animals were taken were, if one can judge from Bottazzi's writings, less concentrated than that from which his specimens were obtained; if any adjustment to aqueous media were to take place it would be in the direction opposite to that indicated by the above figure. It is certainly true, however, that the blood of fresh water and land turtles shows a depression of the freezing point which is distinctly less than that of marine turtles; Bottazzi found Δ for *Emys europa* $= -0.463^\circ$ to -0.485° C., while for *Pseudemys elegans* of the Mississippi Valley we obtained a depression in which $\Delta = -0.48^\circ$ C. These figures are so much below those obtained with the blood of marine turtles that they would seem to indicate the possibility of some degree of adjustment to the concentration of the external medium; on the other hand it is a fact that the land turtles do not show a more concentrated blood than do those which live mainly in fresh water.

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[1] The author is indebted to Mr. Hay for the identification of these animals.

This point was put to the experimental test upon the marine turtles, *Colpochelys kempi* and *Caretta caretta*; the last figure in the previous data given above for each of these species was obtained, with the blood of a specimen which had been kept for two months in a tank containing fresh water. There was absolutely no change in the concentration of the blood of these individuals, and we feel justified in concluding that adjustments of the nature of those under consideration do not take place in these forms at least not within the duration of our experiments.

(*d*) *Fresh Water Fish.*—Preliminary to a study of the effects of osmotic and saline media upon fresh water fish (soon forthcoming) it was desirable to know the osmotic pressure of the blood of forms taken from the Mississippi river. The following list contains some forms peculiar to this region on which no data have hitherto been given.

1. *Polyodon spathula* = − 0.492°, − 0.486°, − 0.50° C.
2. *Scaphirhynchus platyrhynchus* = − 0.505°, − 0.507°, − 0.503° C.
3. *Lepidosteus osseous* (L.). ("Gar") = − 0.487° to − 0.52° C.
4. *Amia calva* (L.) (land locked) = − 0.508° C.
5. *Catostomus teres* = − 0.51° to − 0.52° C.
6. *Perca fluviatilis* = − 0.498° to − 0.51° C.

Fresh water ganoids are seen to have blood which is identical in concentration with that of fresh water teleosts. All have blood less concentrated than that of any of the marine fishes and it is conceivable that in the case of these animals some adjustment to environment has taken place; such adjustments are known for marine fish as has been shown by the author (1) and others (Frédericq, Bottazzi, Dekhuysen (8), Dakin, *loc. cit.*).

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Note.—For other literature consult the papers by Garrey[1] and by Scott;[7] also Bottazzi, Ergebnisse der Physiologie, 1908, VII., p. 162; cf. also Table I. of this paper.

CONCERNING BRACHET'S IDEAS OF THE RÔLE OF MEMBRANE FORMATION IN FERTILIZATION.[1]

JACQUES LOEB.

1. A recent publication by Brachet[2] seems to make it necessary to discuss once more the relation between membrane formation and development. The writer had shown in 1895 that if oxygen is completely withdrawn from the fertilized sea urchin egg no development is possible, while the moment oxygen is admitted the development can begin again. As he had suggested in 1906 and as has since been proved by O. Warburg, and H. Wasteneys and the writer, the entrance of the spermatozoön into the egg of the sea urchin increases the rate of oxidations in the latter (by 400 or 600 per cent). The entrance of the spermatozoön causes also a membrane formation which is very marked in the fresh egg and is generally less marked or may appear to be absent if the egg has been lying in sea water for a day or more. It has been shown, moreover, that the artificial production of a membrane in the unfertilized egg by butyric acid has the same influence upon the increase of the rate of oxidations as the entrance of the spermatozoön. These and other facts seemed to support the view of the writer that an alteration of the surface of the egg, which usually but not necessarily results in a membrane formation, is an essential feature of the development of the egg.

More recent experiments by Warburg[3] have made it very probable that the process of oxidations in the sea urchin egg is a case of catalysis by iron, which is confined mainly if not exclusively to the surface; and this fact, in connection with the data mentioned above, seems to indicate that the process which underlies membrane formation in the unfertilized egg may consist in bringing about or rendering possible the iron catalysis which is responsible for the sudden increase in the rate of oxidations after artificial or natural membrane formation. Since fertilization by sperm is accompanied by a membrane formation and followed by the same increase in the rate of oxidations as is artificial membrane formation by butyric acid, it is probable that the alteration of the surface (underlying membrane formation) is also the cause for the increase in the rate of oxidations in the process of natural fertilization.

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[1] From the Rockefeller Institute for Medical Research, New York.

[2] *Compt. rend. l'Acad. d. sc.*, CLIX., 642, 1914.

[3] Warburg, *Zeitschr. f. physiol. Chem.*, XCII., 231, 1914.

2. The writer has time and again stated that the formation or non-formation of the fertilization membrane is of only secondary importance; what matters are the physicochemical changes which underlie the membrane formation and which are responsible for the sudden rise in the rate of oxidations of the sea urchin egg after artificial or natural membrane formation; and which may even occur when for some reason the fertilization membrane is modified or when its formation is entirely suppressed. There is no doubt that in the writer's first experiments with the purely osmotic method, the fertilization membrane was often very indistinct or in some cases even completely lacking, while nevertheless the enormous increase in the rate of oxidations and development to the pluteus stage ensued.[1]

It is possible to modify the surface of the unfertilized egg in such a way that if it is later fertilized by sperm the abnormal character of the membrane formed, or the abnormal conditions of the surface, may lead to the death of the egg. The writer described such a case in 1909.[2] When the unfertilized eggs of *Strongylocentrotus* were treated for five minutes with a hyperalkaline solution of NaCl (50 c.c. $m/2$ NaCl + 1.0 c.c. N/10 NaOH) and then transferred to normal sea water to which sperm was added, the eggs were all fertilized but apparently without membrane formation, though in reality probably with a tightly fitting membrane. They all segmented but perished in the blastula or gastrula stage. When, however, the eggs were not fertilized immediately after the treatment with alkali but after they had been in the sea water for one hour or more, a more normal membrane was formed and the eggs developed into plutei. Why did the eggs only live to the blastula or gastrula stage when they were fertilized immediately after the alkali treatment? Should this have had something to do with the abnormal character of the membrane which was formed when the egg was fertilized immediately after the alkali treatment? Were the cells pressed by the membrane which was too tight, and did this pressure kill them if prolonged? If this were the case, a tearing of the membrane should save the life of the egg. It would be of interest to try this experiment.

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[1] The literature of the subject can be found in the writer's recent book on "Artificial Parthenogenesis and Fertilization," Chicago, 1913.

[2] Loeb, "Die chemische Entwicklungserregung des tierischen Eies," Berlin, 1909, p. 117.

3. In a recent number of the *Comptes rendus de l'Académie des Sciences*, Brachet has published an observation which may or may not be similar to the one just mentioned. He found a year ago that if the eggs of the sea urchin, *Paracentrotus lividus*, at Roscoff, are put for two hours in contact with sperm of *Sabellaria alveolata* (which cannot fertilize the eggs) and if they are afterwards fertilized with sperm of their own species, they develop without apparently forming a fertilization membrane. From this Brachet concludes that the formation of a fertilization membrane is not necessary for development, a conclusion which will surprise nobody who is familiar with my first experiments on artificial parthenogenesis, or who has ever fertilized eggs which have been lying in sea water for several days. Moreover, Brachet observed that the sea urchin eggs which are fertilized with sperm of their own species, after two hours' treatment with the sperm of *Sabellaria*, die at the time of gastrulation. The prolonged treatment of the eggs of *Paracentrotus* with the sperm of *Sabellaria* seems therefore to have a similar effect as the short treatment of the egg of *Strongylocentrotus* with the alkaline NaCl solution in my experiments.

4. The deductions which Brachet draws seem, however, difficult to reconcile with each other. We stated already that he assumes that the eggs of *Paracentrotus* after two hours' treatment with the sperm of *Sabellaria* form no fertilization membrane after fertilization with their own sperm. Yet, he states further that these eggs die in the gastrula stage for the reason that they cannot hatch; for if he shakes the eggs and thereby destroys "la couche

corticale" the larvæ can hatch and are now able to develop into plutei. The only membrane, however, which can prevent the eggs from hatching is the fertilization membrane, and it is impossible to harmonize the two statements of Brachet, first, that these eggs have no fertilization membrane and, second, that the gastrulae cannot hatch unless the membrane of the egg is pierced. Professor Goldschmidt, to whom I showed Brachet's paper suggested that Brachet probably means by "couche corticale" the hyaline membrane (Herbst's "Verbindungsmembran") which surrounds the blastomeres and that he assumes erroneously that this hyaline membrane forms a continuous layer around the blastula in the same way as the fertilization membrane does. This is, however, not the case since the hyaline membrane participates in the process of segmentation and forms a distinct layer around each individual blastomere, but not a continuous envelope around the whole blastula.

Brachet's observation is intelligible on the assumption that the egg after it has been treated with the sperm of *Sabellaria* forms a very tightly fitting membrane when it is fertilized with its own sperm and that this membrane must be torn by shaking the egg in order to allow the blastula to hatch (or to escape from being killed by the mechanical pressure of the tightly fitting membrane?). Brachet found also that it is possible to substitute for the shaking of the egg a treatment with butyric acid, which as he assumes also tends to remove the obstacle to the hatching. This may be correct, but unfortunately he draws the further conclusion that the butyric acid treatment must have the same effect upon the unfertilized egg as upon the fertilized egg which has previously been treated with the sperm of *Sabellaria.* Leaving aside the fact that the unfertilized egg has no membrane, it has been shown that the butyric acid treatment raises the rate of oxidations of the *unfertilized* egg about 400 or 600 per cent., while acid does not increase, but, on the contrary, lowers the rate of oxidations in the *fertilized* egg. Moreover, the writer has shown that if a *fertilized* egg is treated with butyric acid, in the same way as is required for inducing artificial parthenogenesis, the *fertilized* egg is not injured, while the inducing of a membrane formation by butyric acid in the *unfertilized* egg leads to the rapid death of the latter, if it is kept at room temperature and if it does not receive a second treatment either with a hypertonic solution or lack of oxygen. This case was fully discussed by the writer in a recent paper.[1] It is therefore not justifiable to conclude that the action of butyric acid on the unfertilized egg must be identical with the action of the same substance on a fertilized egg, treated beforehand with the sperm of *Sabellaria.*

Should it be possible that Brachet's "couche corticale" is the chorion or the "jelly" which surrounds the unfertilized egg? But this jelly is normally dissolved when the egg is fertilized. It might be conceivable that the sperm of *Sabellaria* causes a hardening and a contraction of this jelly which protects it against being dissolved by the sperm of the sea urchin and that subsequent shaking or a subsequent treatment with acid destroys this jelly. But granted this were the case, it would be erroneous to use experiments on an artificially altered chorion to draw conclusions upon the rôle of membrane formation in fertilization or artificial parthenogenesis.

The writer wonders how Brachet (or Herlant) are going to harmonize the following well-established facts with their views. If the eggs of *Strongylocentrotus purpuratus* are treated with hypertonic sea water for about two hours, they form in most cases no membrane and nothing happens to them except that a certain percentage of them begin to divide very regularly into 2, 4, 8, possibly 12 or 16 cells and then stop. Such eggs are to all appearances in the resting stage and live as long as the other unfertilized eggs if nothing is done to them. If they are fertilized by sperm each blastomere forms a special fertilization membrane and now each blastomere develops into a blastula or into a pluteus, according to the size of the blastomere. They also develop into plutei if an artificial membrane formation is called forth with the aid of butyric acid. The writer is inclined to explain this phenomenon by assuming that the treatment with the hypertonic solution called forth two effects, one of which was a peripheral change resulting in an increase in the rate of oxidations. This effect is, as the writer has shown, reversible and was possibly reversed while the eggs were in an early stage of development. It seems to the writer impossible to reconcile these observations with the purely morphological views of Brachet or Herlant.

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[1] Loeb, "Weitere Beiträge zur Theorie der künstlichen Parthenogenese," *Arch. f. Entwcklngsmech.*, XXXVIII., 409, 1914.

Brachet (like Herlant) tries to explain the phenomena of artificial parthenogenesis and fertilization without any consideration of the striking chemical processes that accompany fertilization and artificial membrane formation. He reverts to that standpoint of the pure morphologist which Sachs, in his papers on "Matter and Form in Plants" characterized as "empty formalism." This standpoint disregards the sources of energy in life phenomena and treats morphological changes as if they required no source of energy. It seems to the writer that the fact of the necessity of oxygen for development, the fact that mere membrane formation (both by butyric acid or by a spermatozoön) raises the rate of oxidations 400 or 600 per cent, and the fact that the amount of rise is identical in both cases, are so striking, that these facts cannot be ignored in a theory of the rôle of membrane formation in the development of the sea urchin egg. The writer has always considered the changes underlying the membrane formation as the essential factor in the initiation of development, while he considered the formation of a fertilization membrane only as a welcome but not essential indicator of the chemical changes in the surface of the egg; a fact which Brachet, on account of his disregard for the chemical processes, has entirely overlooked. Brachet, from his purely morphological standpoint, erroneously assumes or makes it appear as if I considered the formation of a visible membrane as the only and essential act in the initiation of development.

AN EXPERIMENTAL ANALYSIS OF FERTILIZATION IN PLATYNEREIS MEGALOPS.

E. E. JUST.

Study of the breeding habits of *Platynereis megalops* revealed the fact, as has been pointed out (Just, '14), that insemination takes place in the body cavity of the female and that although egg laying begins often but five seconds after copulation, the eggs will not fertilize when artificially inseminated after exposure to the action of sea-water. It is this failure of sea-water insemination that forms the basis of the present contribution to the analysis of fertilization in *Platynereis*. In order clearly to interpret the phenomena of sea-water insemination a study of the morphology of the normal fertilization was made (see Just, '15*a*).

The experiments undertaken for the analysis of fertilization in *Platynereis* come under three heads:

A. Conditions of successful insemination.

B. Cross fertilization with *Nereis*.

C. Artificial parthenogenesis with various agents.

B and C are taken up mainly because they supplement results under A.

A. Conditions of Successful Insemination.

During the summer of 1911, I was studying the maturation and fertilization of the *Platynereis* egg for comparison with those processes in *Nereis*. The methods of insemination used with *Nereis*, cutting out the eggs and sperm in sea-water, gave no cleavage. Various trials with the utmost care, using diverse methods never gave cleavage. Not until August 24, 1911, did I chance to find that normally insemination takes place in the body cavity of the female (cf. Just, '14).

1. *Observations on Eggs Inseminated in Sea-water.*

If eggs and sperm be cut out of *Platynereis* and mixed in sea-water, the phenomena of maturation, sperm attachment, and copulation of the germ nuclei may be readily followed; but such eggs do not segment nor do they ever develop into swimming forms.

The Living Egg.

If insemination be made in a suspension of India ink ground up in sea-water, the jelly formation may be easily followed: it differs but little from the cortical outflow observed in eggs normally laid. All eggs, however, do not secrete this jelly; of these, some remain in the germinal vesicle stage and others go through maturation with all or part of the cortex intact.

As in the normally inseminated egg (see Just, '15*a*) no cone is present. More often than in the normally laid egg a broad plateau of cytoplasm marks the point of sperm attachment. The sperm, from one to six, are attached to the membrane above this raised cytoplasm or near it.

Maturation proceeds about as in the normal egg. At maturation stages slightly later than in the normal egg, the sperm may be found in the egg. It moves forward with aster formation. The pronuclei meet, remain apposed for a short time, separate, and fade from view. This is not true of all eggs; for apparently, those in the germinal vesicle stage or in maturation stages with cortex intact never engulf the sperm. Moreover, in many eggs that are in maturation with the cortical layer gone, one cannot find sperm.

These eggs never divide. At first, 1911, I thought that this behavior of the egg was due to injury of the worms. Its significance became clear only after the discovery of the normal method of egg-laying.

The Sectioned Egg.

During four seasons eggs have been preserved at three and five minute intervals upward to two hours after insemination in sea-water. Study of the sectioned eggs confirms the findings of the study of living eggs. Many eggs remain ovocytes with sperm attached or not. Those that go through maturation do so with or without jelly formation. Eggs that form jelly are likewise of two classes: those in which sperm are found to have penetrated and those in which no sperm are found.

I have not been able so far to determine any structural differences in the ovocytes with and without sperm attached. In the case of the eggs that maturate with the cortex wholly or partially intact, the spindle may be abnormal. In most cases if it reach the periphery of the egg it does so at a point practically devoid of cortical cytoplasm. Or again, it may lie parallel to a tangent of the egg membrane.

Those sections which reveal the sperm within the egg are in the minority. It appears from experiments several times repeated during the four seasons of study that the penetration of the sperm depends upon the amount of sea-water used. If the eggs be inseminated in a large quantity of sea-water or washed (by changing the water several times) very few eggs form jelly. With less water more form jelly. Eggs inseminated quickly in small quantities of sea-water are capable of engulfing sperm.

The history of the penetration as known may be briefly given. One finds sperm external to the egg at different stages. How it gets into the egg I cannot yet state with certainty although this point has received most careful study for three years. Material has been prepared in every way possible to demonstrate the early penetration. So far I have not found the sperm entering the egg as a slender thread like that in the normal egg. It can be easily demonstrated in the endoplasm. On one slide of the 1911 series, for instance, I counted twenty sperm heads with their asters lying near the centre of the egg. The sperm head remains for a longer time than in the normal egg a black knot with a long drawn out thread extending to the single aster. A second aster has never been found. The germ nuclei copulate but the eggs never cleave. Various stages are found from sixty to one hundred twenty minutes after insemination—sixty minutes after cleavage in the normal egg. The pronuclei after apposition gradually separate and degenerate as discrete nuclear masses. Many eggs show only one chromatin mass in process of degeneration; doubtless, these are eggs which sperm do not enter. The sections of such eggs closely resemble those of *Nereis* eggs from which the sperm have been removed (see Lillie, '12). I have repeatedly made observations on living eggs inseminated in sea-water and on sections. I have yet to find a single cleaving egg.

Two hours after insemination the eggs exhibit cytoplasmic stratification; the oil drops later fuse to form one at the vegetative pole. Twelve hours after insemination the conditions are the same; there is never a swimming form among these eggs.

2. *Nature of the Inhibition to Development.*

It may be very clearly shown that sea-water is responsible for the lack of cleavage by the method of "dry insemination." If males and females dried on filter paper be cut up separately and the drops of eggs and sperm thus obtained be mixed with subsequent addition of sea-water, a percentage of the eggs always cleave and develop into normal trochophores. I have kept larvae from such dry inseminations until they were seven mm. long with thirty or more segments, few differing from normally laid eggs. There is doubtless an optimum time after mixing for the addition of sea-water, but any time upward to two minutes gives results. The following is an example:

August 3, 1912. To determine the time interval after mixing dry eggs and sperm before adding sea-water.

Water Added.	Per Cent. of Cleavage.
1. At once	60
2. Five seconds after	50
3. Ten seconds after	90
4. Twenty seconds after	45

Practically, as soon as eggs and sperm are mixed, sea-water may be added. I have not been able to add sea-water quickly enough after mixing to prohibit cleavage. If the eggs are allowed to stand two minutes the majority are plasmolyzed by the addition of sea-water.

The amount of sea-water that will permit fertilization has been repeatedly determined:

July 28, 1912, 9:45 P.M. Experiment to determine the maximum amount of sea-water that permits fertilization.

Males and females are thoroughly dried on clean filter paper. A male and a female placed in each of the eight perfectly dried clean watch glasses. Sea-water added as follows:

No. 1	1 drop.
" 2	2 drops.
" 3	3 "
" 4	4 "
" 5	5 "
" 6	6 "
" 7	10 c.c.
" 8	no sea-water.

The worms were then cut up and flooded with sea-water, later transferred to fresh sea-water in finger bowls.

Nos. 1, 2, 3 and 8 gave cleavage; a per cent. of normal trochophores was found the next morning. In dishes 4, 5, 6 and 7 not an egg divided, no swimming forms developed.

No single observation in the whole work was made as often as this; the results are wonderfully precise. As I shall show later the experiment quoted was conducted under the optimum conditions, and yet it shows the inhibiting effect of such a surprisingly small quantity of sea-water. All other observations show two drops of sea-water for each worm to be the maximum that will permit normal fertilization. In no case have I got cleavage where two and one-half drops of sea-water for each worm (*i. e.*, five drops to two worms) were used. While the same pipette was used to secure equal drops, the worms, females particularly, vary in size. I have usually taken the average females for these experiments. Such an animal, as found by actual count in three cases, has about 11,000 eggs. There is enough variation, however, in the size and weight of the worms to make impossible any law concerning the lethal amount of sea-water. I believe, nevertheless, that there is an optimum time for the addition of sea-water—equal to the time the sperm are in the female in normal insemination; and an optimum amount of sea-water—about as much as the worms will take up after thorough drying.

The results of these inseminations over a period of four seasons prove clearly that sea-water except in minute quantity is fatal to fertilization.

Does Sea-water Injure Egg, Sperm, or Both?

Three explanations of the failure of *Platynereis* eggs to cleave after insemination in sea-water are possible:

(*a*) Both eggs and spermatozoa are injured by the sea-water.

(*b*) The sperm alone are injured by the sea-water.

(*c*) The eggs alone are injured by the sea-water.

The failure of the eggs to go beyond maturation may be due to the injurious action of the sea-water on both eggs and sperm alike. It would seem reasonable to assume that for internal insemination both cells need the perivisceral fluids. It might be difficult to conceive how this adaptation in *Platynereis* could have taken place acting on one only of the sex elements. As both eggs and spermatozoa are protected by body fluids in normal insemination, so both are exposed to the lethal action of sea-water. Embryologists are all careful when inseminating eggs of forms in which insemination normally taken place in the sea not to contaminate the dishes containing ova with the animal's tissues or fluids. Lillie ('13*b*, '14) has shown why this is essential. I have, however, repeatedly with success fertilized *Nereis* eggs dry (see Just, '15*b*) doubtless because the body fluid of *Nereis* is practically negligible. And the case of *Platynereis* is similar to that of *Nereis*; in this smaller worm there is no more fluid; the female is a mere locomotor ovary, although the male does have a small amount of fluid and a great number of corpuscles.

The second possibility is that the sperm alone are injured by the sea-water. Injury to the sperm through transference from the male's body fluid to sea-water, however, cannot be due to difference in osmotic pressure. For as Frédericq has shown, and Garrey since for the Woods Hole region, the osmotic pressure of invertebrate body fluids is about the same as that of sea-water. Moreover, *Platynereis* sperm in sea-water as far as I could determine exhibit none of the effects experimentally produced by Koltzoff on various sperm cells including those of *Nereis* (*dumerilii?*) through treatment by various salt solutions or those conditions described by de Meyer with hypotonic and hypertonic solutions. In some other way, then, the sperm must be assumed to be weakened but still capable of partially fertilizing the egg as the Hertwigs, Gemmil, Budington, Dungay, etc., have shown. And indeed my *Platynereis* slides of sea-water inseminated eggs show similarities to the figures by Lillie of the penetration of injured sperm in *Nereis*; in *Platynereis*, however, the germ nuclei develop a little farther. Steinach long ago, later Walker ('99, '11) and Hirowaki have shown that in mammals the prostate secretion is necessary for fertilization. Sea-water, then, might injure the sperm and hinder fertilization by destroying a supporting medium necessary for fertilization. (On this point, cf. Gemmil's experiments.)

Finally, a third explanation is possible: the egg alone is injured through sea-water treatment. The egg, in this case, may be dependent on a substance in the female's body or on some secretion of its own necessary for fertilization. Both egg and sperm may need body fluids but sperm may be hardier, egg less resistant.[1]

The seasons of 1912 and 1913 were largely given over to experiments to determine which possible explanation is valid for *Platynereis*. In 1914, many of these experiments were repeated. And I may say at once that the explanation must come under the third head as shown by the following experiments.

The Experiments.

The plan of the experiments is briefly as following:

Males and females were cut up separately in dishes of clean sea-water. The bits of tissue were carefully removed, the dish of eggs being handled with utmost care to prevent unnecessary agitation. The eggs and sperm suspensions were filtered after having remained in sea-water for varying lengths of time. Sexual products treated thus are designated "washed eggs" and "washed sperm."[2]

Males and females were thoroughly dried on filter paper or clean sheer linen. The males were cut up in dried clean watch glasses; the females were cut up in the same way or pricked when most of the eggs that escaped were collected in dry watch crystals. Bits of tissue were always removed. Such eggs and sperm are "dry eggs" and "dry sperm."

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[1] That the resistance of eggs and sperm of both *Nereis* and *Platynereis* is unequal would seem probable from the following: If to a *Nereis* sperm suspension janus green be added the fertilizing power of the sperm is in no wise impaired; or if the dye be added to sea-water the living males absorb it readily without any injurious effect on the sperm. The same quantities of the dye in sea-water is toxic to the egg before or at insemination. Eggs taken from a female *Platynereis* that has been swimming in a janus green-sea-water solution that is not toxic to the males or their sperm will not fertilize. Cf. also action of nicotine on *Strongylocentrotus* sperm and eggs as observed by the Hertwigs.

[2] Several methods were used for "washing" sperm and freeing them of sea-water, among others that of centrifuging at high speed for six minutes. These were all abandoned for the method here described.

For a given experiment eggs and sperm were mixed and after an interval of time varying from five to sixty seconds flooded with sea-water. Four kinds of inseminations were made:

Washed eggs × *washed sperm.*
Washed eggs × *dry sperm.*
Dry eggs × *dry sperm.*
Dry eggs × *washed sperm.*

The experiments fall into two groups: "A.M. inseminations"—made the morning after the worms were captured; and "P.M. inseminations"—made during the evening of capture.

The following table gives a summary of results:

TABLE I.

Eggs.	Sperm.	Group.	Development.
Washed	Washed	A.M. and P.M.	None.
Washed	Dry	A.M. and P.M.	None.
Dry	Dry	A.M. and P.M.	Cleavage and larvæ.
Dry	Washed	A.M.	None.
Dry	Washed	P.M.	Cleavage and larvæ.

Washed eggs, inseminated with dry or washed sperm, never reach cleavage stages nor do they ever produce swimming forms.

I have commented above on the *dry egg* × *dry sperm* series. These eggs cleave and later produce normal larvae.

Washed sperm × *dry eggs* of the A.M. group (1912) did not yield cleavage or swimming forms. The worms do not thrive well in the laboratory. The practise, therefore, of conducting experiments the morning after capture has been since 1912 practically abandoned. The only test for the vitality of the worms is copulation—a test the very nature of which precludes experiment. Doubtless, therefore, this set of experiments gave no results because the animals were not fit. Study of sections of eggs normally inseminated and laid as early as 5 A.M. shows a large percentage in the germinal vesicle stage. I have made counts in dishes of living eggs to show at the later cleavage stages the proportion of eggs still in the germinal vesicle stage. For example, August 8, 1912, 2 P.M., six hours after laying of 10,851 eggs (from one female) six per cent. were still in the germinal vesicle stage. Other counts of living eggs and of sections show higher percentages. Every egg laid the night of capture cleaves. Dry inseminations, day or night, at best never give more than ninety per cent. of cleavages. The poor quality of the animals after several hours in the laboratory may account for the failure of the *dry eggs* × *washed sperm* A.M. group to cleave. But since the *dry eggs* × *dry sperm* A.M. series gives cleavage, I am rather inclined to believe that the method used was poor: for instance, the filter paper then used was too soft allowing the loss of most of the spermatozoa or too much water was left when the dry eggs were added.

The results with *dry eggs* × *washed sperm*, P.M. group are wonderfully uniform and show conclusively that the sea-water, at least for the exposures used, has no harmful effect on the sperm. The method used is simple. As soon as possible after capture one to three males are cut up in from 8 drops to 20 c.c. of sea-water and allowed to stand upward to twenty minutes. (The sperm are active after having been in sea-water for twelve hours.) The sperm suspension is then filtered. I used a very hard filter paper. This paper was then tilted and thoroughly drained until under the lamplight the glistening water was thoroughly absorbed. A dried female was cut up on the filter paper or pricked and the eggs thus procured rolled over the paper to reach the sperm left behind or caught in the pores of the filter. The whole was then put in a dish of clean sea-water. It would be tedious to cite the individual experiments. They show conclusively that dry eggs inseminated with washed sperm develop in normal fashion.

Now since, as has been shown above, there is a minimal amount of sea-water that will permit fertilization, dry eggs ought to fertilize if put on the filter paper before all the water has been absorbed. Such indeed is the case. Moreover, dry eggs put in two drops of thin sperm suspension develop. From a suspension made by cutting up one or more males in sea-water two drops are taken. Dry eggs put in this cleave and next morning swim.

This observation led to a series of experiments (during 1913 and 1914) designed to ascertain whether or not the density of the sperm suspension is a factor in the fertilization of *Platynereis*.

These experiments prove in general that the number of dry eggs added to sperm suspensions that develop depends upon the density of the suspension. The denser the suspension the larger the number of trochophores. Moreover, for dense suspensions the minimum amount of sea-water permitting fertilization appears to be slightly higher than for thin suspensions. Cleavage is directly a function of the chances of the spermatozoa reaching the egg before the fertilizing substance is lost.

The time of flooding with sea-water after insemination is also important for the highest percentage of cleavage. But these factors cannot be expressed with mathematical exactness. Some points, particularly with reference to inseminations with dense suspension need further experiments to determine their significance.

That the egg when exposed to the action of sea-water quickly loses something necessary for fertilization must be the conclusion drawn from these experiments with washed or unwashed eggs. Even *thirty seconds* residence in sea-water, as repeatedly proved, is sufficient to inhibit cleavage in every single egg. If dry eggs from a single female be put in five cubic centimeters of sea-water and thoroughly drained as soon as they settle they will not develop after insemination although this procedure may take but a half minute. The egg alone is affected by sea-water; the fertilizing power of the sperm is not affected by exposure to sea-water.

3. *The Nature of the Fertilizing Substance.*

The fertilizing substance once lost cannot be restored. If washed eggs be mixed with an extract obtained by crushing dry eggs in one or two drops of sea-water and dry sperm added, cleavage does not result. I lay no stress on this, however, for it seems to me that such an extract might yield anything.

The presence of various substances in the sea-water or the lowering of the temperature of the sea-water does not prevent or restore the loss of this substance.

KOH.—Eggs were teased out of the female directly into sea-water plus KOH in various proportions. Or, eggs from dried females were placed in the solution. After remaining from thirty seconds to two minutes in the alkaline sea-water the eggs were inseminated dry and flooded with sea-water. In other cases inseminations were made in the solutions. Washed eggs were similarly treated. Whatever the method alkaline sea-water never gave cleavage. (Cf. sections on cross fertilization and artificial parthenogenesis.)

Hypertonic and Hypotonic Sea-water.—Eggs, both washed and dry, were treated with 2½ M KCl + sea-water as follows:

1. 1 drop 2½ M KCl + 19 drops of sea-water.
2. 2 drops " " + 18 " " "
3. 3 " " " + 17 " " "
4. 4 " " " + 16 " " "
5. 5 " " " + 15 " " "
6. 6 " " " + 14 " " "

Dry sperm were added at once and the dishes flooded with sea-water after five minutes. Or, after treatment for varying number of minutes the eggs were inseminated dry. The eggs developed no farther than with KCl treatment alone (see beyond); they form jelly and maturate.

Hypotonic solutions used similarly gave no cleavage.

Ether.—The following table is a summary of the experiments with ether:

Eggs.	Solutions Used.	Exposure.	Inseminations.
Washed,	.3 to .6 per cent.	1 to 5 minutes dry;	in the solution.
Dry,	"	" "	" " "
Teased,	"	" "	" " "

"Teased" eggs are those got by cutting up the female in the ether-sea-water.

A few eggs form jelly and maturate after the ether treatment. Compared with sea-water inseminations, ether cuts down the per cent. of maturations. According to R. S. Lillie ('12) starfish eggs resistant to fertilization may be rendered normal by ether in low concentration. In *Platynereis* the condition is different. The egg is not rendered resistant to fertilization by the action of sea-water; it is weakened through loss of something by the sea-water since it combines but feebly with the sperm The ether as in *Asterias* renders the *Platynereis* egg irritable since as shown by the low percentage of maturation more fertilizing substance must be secreted.

KCN.—Inseminations made with washed or dry eggs during or after treatment with KCN (1 per cent. KCN and sea-water made in various proportions) gave only maturation. But the eggs will maturate in KCN alone while in the solutions. (Cf. Allyn on *Chœtopterus.*)

$CaCl_2$.—Newman found that $CaCl_2$ inhibits fertilization in *Fundulus* through a precipitation effect. I thought that in somewhat the same way calcium chloride might through action on the cortex inhibit the loss of the fertilizing substance in *Platynereis.* M/2 $CaCl_2$ added to sea-water in different quantities does not inhibit the loss of the substance since after the calcium chloride treatment the egg does not fertilize.

Cooled Sea-water.—Sea-water was cooled to 10.5° C. and dry eggs after 30, 60 and 90 seconds' treatment in 5 c.c. were inseminated at this temperature or after the cooled water was pipetted off. In some experiments the female was kept at the low temperature for several minutes before the eggs were cut out. 5 c.c. of sea-water were used in each experiment. The eggs never cleave, but more form jelly and maturate than controls inseminated in ordinary sea-water. This would seem to indicate a slowing down of the secretion. The effect of cold is just the opposite of the effect of ether. Unfortunately, only few of these experiments were made. Perhaps they should be repeated at lower temperatures.

Concerning the nature of this substance, some of my earliest notes are of interest. After insemination in sea-water I found some time later (forty minutes in one case) "sperm dancing above the eggs." In 1914, I found the sperm of sea-water insemination active after twelve hours. One does not find this after dry insemination, even with excess of sperm. Sperm in the dishes of successfully inseminated eggs are profoundly changed. Study of the movements of *Platynereis* sperm reveals the circular swimming of echinid spermatozoa, as shown by Buller, Gemmil, Winslow, and others (see also Dewitz, Ballowitz, etc.). They finally become quiescent through lack of oxygen[1] in various positions without orientation. After dry inseminations they come to rest, as can be seen after flooding the dishes, definitely oriented and not in haphazard arrangement. Clustered among the jelly hulls, their heads point toward the eggs. On occasions, I believed that I demonstrated the agglutination of the sperm by sea-water in which the eggs had been lying. The evidence is not clear-cut and more recent attempts have failed. The egg charged sea-water, however, does activate the sperm.

I wish to point out the serious difficulties experienced in the series of sperm agglutination experiments. In the first place, twenty "large" dried males (two and one half centimeters long) do not yield enough sperm and body fluid to make up a drop as large as a drop of dry sperm from a very small *Nereis*. Then again the thickest suspension got is largely made up of blood corpuscles. I have never succeeded in procuring a "milky suspension"—the admixture of corpuscles and body fluid giving always a pinkish mixture. And finally, one cannot always get twenty or more males necessary to make up even this thin sperm suspension. Repeated efforts, therefore, extending through two seasons have not been marked with very positive results.

With *Nereis* sperm, the case is indisputable. If water in which *Platynereis* have laid eggs be taken it is found to have an agglutinating effect on *Nereis* sperm. Thus:

August 18, 1914. At 10:15 P.M., ten females laid eggs in six c.c. of sea-water each. After five minutes some of this water was drawn off—20 c.c. in all. *Nereis* sperm suspensions were made up fresh at 10:20, 10:30, 11:00 and 11:05. A drop of the sperm suspension was mounted on a slide under a raised cover slip. A drop of the water taken from the dishes of eggs was injected beneath the cover slip. Under the microscope, the quiescent sperm appeared at first intensely active, then rushed together and formed agglutinated masses among others still free-swimming.

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[1] This fact was brought out in 1913 when I was repeating some old observations on echinoderm spermatozoa. While experimenting with the sperm of *Thyone* in janus green solutions, I noted after some time had elapsed that cover-slip preparations showed that bacteria present previously bluish in color had changed to a decided red. Later observations proved that as the dye was reduced in bits of tissue under the cover slip the sperm quieted down in various positions.

The same experiment succeeds if one uses the water from dishes in which uninseminated eggs have remained for a few minutes. Washed eggs do not cause agglutination of *Nereis* sperm; water charged by normally inseminated eggs or uninseminated eggs retains its power of agglutinating *Nereis* sperm after twelve hours at least, the reaction coming on more slowly. The freshly charged water acting on fresh sperm suspension gives a clear-cut and beautiful reaction.

It may seem far-fetched to argue that the fertilizing substance lost by *Platynereis* eggs when exposed to sea-water is agglutinin or fertilizin as discovered by Lillie in *Nereis* and *Arbacia* because the washed egg, no longer fertilizable by its own sperm, can not sufficiently charge the sea-water to agglutinate *Nereis* sperm. Yet I believe this is the case precisely. The agglutination of *Nereis* sperm by *Platynereis* egg-water is correlated with jelly formation in *Platynereis* by *Nereis* sperm. In sea-water inseminations, *Nereis* spermatozoa are almost as effective as those of *Platynereis*. Added to this is the difference in behavior of *Platynereis* sperm in egg charged sea-water, in sea-water inseminations, and in dry inseminations.

The evidence may be scant, but it seems to me sufficient to indicate that the substance lost which is necessary for fertilization is identical in nature with the fertilizin of Lillie.

B. CROSS FERTILIZATION WITH NEREIS.

I have mentioned (Just, '14) the fact that it is generally taken for granted that reciprocal crossing of *Nereis* and *Platynereis* is the rule. This led me to attempt cross fertilization. Cross fertilization never produces segmentation or development though it may induce the maturation process.

Of the methods used in echinoderm hybridization—those of Loeb, Tennent,[1] etc.: (1) high temperature; (2) treatment with fresh water; (3) treatment with alkalis; (4) allowing the eggs to stand; and (5) polyspermy—all were tried except the first. Since the eggs of *Platynereis* are normally inseminated in the body cavity and therefore with little sea-water, I tried "dry inseminations": *i. e.*, *Nereis* males were cut up dry and a drop of the sperm without the addition of sea-water added to eggs of *Platynereis* cut up dry. Inseminations were made in a variety of ways as the following table of method shows:

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[1] Dr. Tennent in 1912 very kindly communicated to me at length his latest methods in echinoderm hybridization.

TABLE II.

SUMMARY OF INSEMINATIONS MADE IN 1911, 1912, 1913, AND 1914

Platynereis sperm	on	*Nereis* egg.
1. Few sperm in sea-water.		Fresh eggs in sea-water.
2. Dense sperm suspension.		
3. Few sperm in sea-water.		Stale eggs in sea-water.
4. Dense sperm suspension.		
5. Few sperm, dry,		Fresh eggs dry.
6. Heavy insemination dry.		
7. Few sperm, dry.		Stale eggs washed.
8. Heavy insemination, dry.		

Reciprocal crosses of *Platynereis* eggs and *Nereis* sperm were made.

"Stale eggs" are eggs that have stood in sea-water for several hours. "Stale eggs, washed" are stale eggs on which the water has been changed several times.

These experiments were made repeatedly during four seasons. The sperm of *Platynereis* has practically no effect on the egg of *Nereis* whether fresh or stale, dry or in sea-water. In one experiment (1911) I got jelly formation in a few eggs. This experiment later repeated (1913) gave no result. If *Nereis* eggs be inseminated with *Platynereis* sperm during the evening of capture they show no change the next morning. Inseminated with *Nereis* sperm twelve hours after insemination with *Platynereis* sperm, the eggs develop normally if anything in greater numbers than such stale eggs in ordinary sea-water do.

Nereis sperm will cause *Platynereis* eggs to form jelly, the per cent. of eggs thus responding depending upon the amount of sea-water used and the density of the sperm suspension. But in general many of the eggs fail to form jelly or go through maturation. Many that maturate do so with the cortex partially or wholly intact. Sections of these eggs preserved at three minute intervals after insemination have been studied. The sperm does not enter; or, if it enters must disintegrate early for I have never found sperm nuclei in these preparations.

Clearly, then, one may not use the eggs of these worms indiscriminately.

C. Artificial Parthenogenesis.

The following agents have been used in an attempt to bring about artificial parthenogenesis in the egg of *Platynereis megalops*:

1. Centrifuging,
2. KCl,
3. NaOH,
4. KOH,
5. HNO_3,
6. HCl,
7. Warm sea-water.

The eggs were cut out of the worms in sea-water centrifuged; subjected to varying quantities of salt, alkalis, or acids for different lengths of time; or warmed in sea-water for from five to thirty minutes at 35° C. These methods gave polar body formation, cytoplasmic changes, fusion of the oil drops, and finally chromatin disintegration in the animal hemisphere. The eggs never cleaved.

Study of the literature reveals the fact that the clearest cases of artificial parthenogenesis closely simulating the normal in cleavage and in larval development are of those eggs that have formed one or both polar bodies when shed: the echinids, for example, and the asteroids. Other eggs shed in the germinal vesicle stage like those of *Polynoe* (Loeb '08), *Amphitrite* (Loeb '01; Scott.) *Nereis* (Lillie '11), etc., give only differentiation without cleavage or incomplete cleavage. Loeb and Wasteneys' work on *Chaetopterus* with ox serum as well as Miss Allyn's on the same egg with heat are exceptions. The great exception to the general statement made above is *Thalasema* (Lefevre) where it appears with single substances, acids mostly, normal development is closely simulated. On the whole, however, ovocytes yield less readily to parthenogenetic agents than mature ova.

Mathews' experiments ('01) on *Asterias* may in this connection be cited. He found that when the eggs of this starfish were got while still in the germinal vesicle stage shaking would produce development only after the eggs had remained in sea-water until maturation was gone through with. Sea-water acts as a first stimulus and mechanical shock induces further development. So R. S. Lillie ('08) on the same egg finds that its responsiveness to momentary elevation of temperature as a means of producing artificial parthenogenesis "varies greatly at different periods in the life of the egg." "The most favorable period is some little time (10 to 20 minutes) before the separation of the first polar body."

Reasoning thus, I thought that I might carry *Platynereis* eggs through maturation with one agent and then through cleavage with another. Eggs were, therefore, treated with KCl, KOH, and NaOH in sea-water for various lengths of time and then subjected to heat, shaking, and centrifugal force. In no case did I procure cleavage although the first agent in each case caused maturation. With *Nereis*, on the other hand, KCl and subsequent warming in sea-water induces development (see Just '15*b*).

It is interesting to note that eggs subjected to heat in the minute quantities of sea-water that permit fertilization do not develop beyond maturation. Apparently, the conditions for successful artificial initiation of development are more exacting than those for successful insemination.

We may conclude, then, that the results of attempted cross fertilization and artificial parthenogenesis are harmonious with those of sea-water insemination, so far as cleavage is concerned, in their negative results. The fundamental questions are: (1) the significance of the sea-water insemination and (2) the extent to which the results with *Nereis* sperm and with parthenogenetic agents are capable of like interpretation.

Discussion.

Any analysis of fertilization must deal with the phenomena from the point of view of heredity or of initiation of development. Considered as the process of initiating development, fertilization may be divided into the stages of insemination, sperm penetration, and germ nuclei copulation. As Lillie has repeatedly pointed out[1] experimental evidence must be amassed testing the meaning of each of these stages.

1. Concerning insemination, as Lillie has shown, the egg plays an important part through the production of agglutinins.[2] For both *Arbacia* and *Nereis* it has also been shown that chemotaxis plays a part in insemination. (Lillie, '12, '13*a*, '13*b*, and '14). I believe that *Platynereis* belongs to this class. I may, however, be permitted again to point out the great difficulty attending the use of *Platynereis* eggs on this phase. All the phenomena are extremely rapid, the reactions must be very nice. The material is unfavorable for any intensive study of agglutination and chemotaxis. When one stops to think of the extremely precise reactions of the eggs, one gets a hint of the task. The carrying over of the *smallest* drop of sea-water above the maximum to eggs from vigorous females within the shortest time after capture will prohibit cleavage in every egg.

To answer the general question whether or not eggs secrete substances that activate the spermatozoa, I believe forms whose eggs are inseminated normally in sea-water should be used. So far as *Platynereis* is concerned, agglutination or not, chemotaxis or not, the egg must lose a substance or substances when in sea-water whose presence is necessary for fertilization.

2. Study of the normal fertilization of *Platynereis* indicates that as in *Nereis* the egg plays the active rôle in the penetration of the spermatozoön for it actually draws in the passive spermatozoön. After sea-water treatment I have not, as mentioned above, found the early stages of penetration in eggs fixed at three minute intervals after insemination. Either the sperm penetration is unlike that after normal insemination or penetration takes place with extreme rapidity. In the later stages of penetration it is

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[1] Lectures to classes in embryology, Woods Hole, Mass.

[2] Apparently Buller did not realize that he obtained iso-agglutination of sea-urchin sperm, although he speaks of the sperm forming "balls" and although the phenomena of agglutination were well known at that time. Landsteiner the year before had secured sperm agglutinating sera. Nougouchi's work on *Nereis* sperm is of interest: he demonstrated agglutination with snake venom. The experiments of Schücking, von Dungern, de Meyer, and others are well known. An observation of Walker's ('10) is likewise worthy of mention—the agglutination of the sperm of the rat when mixed with the seminal vesicle secretion of the same animal.

Chemotaxis of sperm has been demonstrated for mammals—see for instance, Löw.

clear that the spermatozoa behave in abnormal fashion even granting that I may have overlooked the amphiaster. The evidence seems to indicate that after sea-water treatment the egg lacks the power to engulf the sperm. However, whatever the method of penetration one point is beyond contradiction: these washed eggs never cleave.

The observations agree with those of Lillie ('14) who notes that some unpublished observations in the case of *Nereis* show that "if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoön, but without causing cleavage of the egg." Miss Allyn found that after KCl treatment of the egg of *Chætopterus*, the spermatozoön may enter but its behavior is not normal. Kite (quoted from Lillie '14) finds that spermatozoa injected into star-fish eggs never give cleavage.

In these cases, the interpretation must be that the "fertilizable" condition of the egg has been destroyed through loss of fertilizin before insemination. In the same way sperm may penetrate unripe eggs as Hempelmann has shown for *Saccocirrus* (so too, von Hofsten for *Otomesostoma* and Shearer for *Dinophilus gyrociliatus*). Two years ago I found that eggs from *Nereis limbata* just before transformation into the heteronereis phase would not fertilize with active sperm either from the nereis or heteroneris form. Moreover, eggs from metamorphosing worms kept for several weeks in the laboratory although apparently ripe would not fertilize on insemination during the dark of the moon. At full moon, sometimes but a few days later, eggs from the same animal would fertilize and develop into larvæ which were kept for weeks. We may assume in these cases that the fertilizin is either absent or is unavailable. Penetration, therefore, may take place before the fertilizable period is reached as well as after it has been passed, but the egg is not capable of fertilization.

3. Apposition of the germ nuclei of *Platynereis* after sea-water insemination may ensue, but never cleavage. After the loss of the fertilizing substance, then, the normal fertilization process may be closely simulated even to the point of the copulation of the pronuclei but development never goes beyond this point. In short, the normal fertilization process demands at the very

outset the fixation by the spermatozoön of the escaping fertilizin. This takes place in *Platynereis* almost instantaneously (see page 93) but brief though this phase may be it cannot be omitted.

The experiments with *Nereis* sperm and agents of artificial parthenogenesis demand explanation. Eggs such as those of echinids used in cross fertilization (Loeb, Tennent, Baltzer, Herbst, etc.) or in artificial parthenogenesis when subjected to treatment are so subjected with their substances intact. They are normally shed in sea-water for insemination and the sea-water does not for some time destroy their fertilizing power. *Platynereis* eggs when subjected in sea-water to foreign sperm or to various agents have lost something through the action of sea-water. This very "something" is necessary for artificial parthenogenesis and, moreover, as shown above (for *Nereis* also) must be present in greater quantity than necessary for fertilization. I am emboldened further to suggest that eggs normally inseminated in the ovocyte stage yield to parthenogenetic agents only with difficulty because they lose fertilizin at the impact of the first stimulus—chemical treatment, shock, etc. Sperm alone, in most cases, are strong enough by fixation of the fertilizin to carry such eggs through their dual phase—maturation and fertilization. Whether by sperm, then, or by artificial agents, the initiation of development is fundamentally the same.[1] The egg plays the leading rôle; it needs but to have its fertilizin activated in order to develop.

The observations on *Platynereis* were rendered less difficult because of the study of the maturation and fertilization in *Nereis*. For this study I was fortunate to be able to supplement my own slides with two series lent me by Professor F. R. Lillie. It is a genuine pleasure here to acknowledge my further indebtedness to him for his many suggestions and for his stimulating interest in the *Platynereis* studies begun at his suggestion and under his direction.

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Wood's Hole, Mass.

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[1] I think that Martin Jacoby's experiments support this view. He found (*Biochem. Zeit.*, *26*, *333–335*) that serum from rabbits into which eggs had been injected showed an increased power to stimulate parthenogenetic development of the eggs. He also found (*ibid.*, *pp. 336–343*) that an enzyme which may be extracted from sperm and from eggs after sperm penetration may be got from parthenogenetic eggs.

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SPOROCYSTS IN AN ANNELID.[1]

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In the summer of 1910, while at work at the United States Fisheries Biological Station, Woods Hole, Mass., I was told by Dr. Gilman A. Drew that what were supposed to be cercariæ had been noticed at different times associated with the annelid *Hydroides dianthus* Verrill among material being used for study at the Marine Biological Laboratory.

Acting upon this suggestion I examined a large number of these serpulids on several dates in August of that year. Although much of the material was examined very minutely, the worms having been removed from the tubes, teased, and everything that even remotely resembled a sporocyst further examined, neither sporocysts nor cercariæ were found.

In the following summer I secured two lots of these sporocysts from this annelid. For the first lot, July 15, I am indebted to Dr. Drew, and for the second, July 21, to Miss Margaret Morris.

In each case the single annelid was lying in a dish of sea water, and in the bottom of the dish there were a large number of sporocysts. These sporocysts were found to contain cercariæ in various stages of development but no rediæ. As they lay free in the sea water the sporocysts were for the most part white, or bluish translucent white. In some of them there were varying amounts of orange pigment of similar appearance to the abundant pigment in the annelid. They were short and thick, bluntly rounded at the ends, and more or less arcuate. In some cases they were curved until the ends almost touched each other. Many of the second lot were orange yellow, also many of them were actively contractile. A frequent change of shape was that from the characteristic short, blunt-pointed sub-cylindrical form to a fusiform shape with elongated and slender-pointed ends. In this condition they were sometimes straight and sometimes arcuate (Fig. 1). In each sporocyst there were tailed cercariæ along with various stages of developing cercariæ, from globular balls of cells, 0.04 millimeter in diameter, to cercariæ, 0.5 millimeter or more in length (Fig. 2). The anterior portion of these cercariæ is sub-cylindrical and slightly tapering at the anterior end. It is marked off from the elongated tail portion by a constriction, which, in the mature cercariæ, is at about the anterior fourth of the entire length. In other words, the tail, which is forked at the end, is about three times the length of the body. Along the dorsal aspect of the body in a few instances a longitudinal row of exceedingly slender spines was noted. As this cercaria resembled very closely a cercaria which I have found in the scallop (*Pecten irradians*), I recorded in my notes that it was likely that these spines, as in the cercaria from the scallop, are remnants of a fin-like membrane, and that the type represented by this cercaria is evidently near that of *Cercaria cristata* Le Val.

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[1] Published by permission of Commissioner of Fisheries.

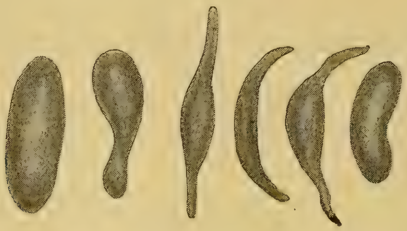

FIG. 1. Contraction shapes of sporocysts, life.

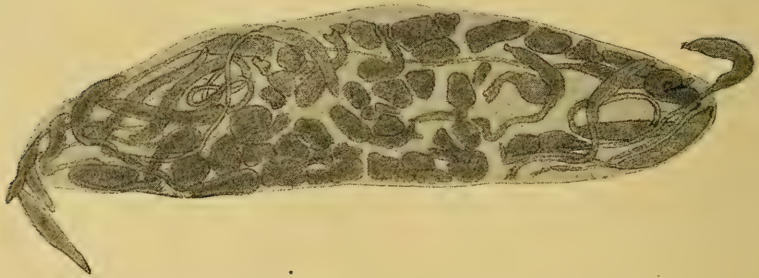

FIG. 2. Sporocyst showing cercariæ in different stages of development. Camera lucida sketch of stained and mounted specimen. Actual length 1.04 millimeter.

On July 19, 1914, through the kindness of Dr. E. J. Lund, I had the opportunity of examining another lot of these cercariæ from this same annelid. Some of these were observed to be covered with an exceedingly thin hyaline membrane which becomes constricted at frequent intervals, the constrictions ultimately being the only part of the membrane that is visible. The cercariæ from *Hydroides*, as was the case with those from the scallop, exhibit great activity, but the nature of their movement is different. Instead of a characteristic pecking motion of the anterior end, the cercariæ from the annelid, occasionally, after lying motionless for a time, perform exceedingly rapid wriggling movements. The anterior end of the body is provided with a short, retractile boring apparatus, shown protruded in Fig. 3.

In addition to the various stages of developing cercariæ, other structures were observed in these sporocysts. With transmitted light these appeared to be granular, but with reflected light, or, with high magnification, they appear to consist of minute oil droplets, at least in part. In some cases they were distributed rather uniformly near the surface, in others they were massed in the central region.

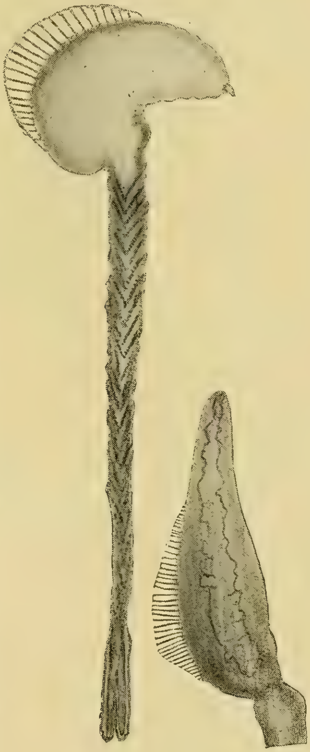

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FIG. 3. Cercaria with fin-like crest. The thin investing membrane is indicated at the posterior end. Length of body 0.17 millimeter, length of tail, 0.52.

FIG. 4. Anterior end of a cercaria with a crest of slender, cilia-like spines.

The number of these sporocysts is very great. In the first lot it was estimated that there were between 900 and 1,000 sporocysts in the dish with the annelid. After the worm had been lying for a few minutes in a dish of clean sea water to which it had been transferred, a number, 25 or more, of sporocysts made their appearance on the bottom of the dish. The worm was then placed in corrosive-acetic and afterwards sectioned. The sections show an immense number of sporocysts (Fig. 5). For the most part they are distributed ventrally in the inner portion of the body wall, although a few lay among the muscles near the exterior, and a few in the epidermis. If I interpret the sections correctly, the sporocysts escape from the ventral side of the serpulid, where the body wall is comparatively thin, and where the sporocysts are in greatest numbers.

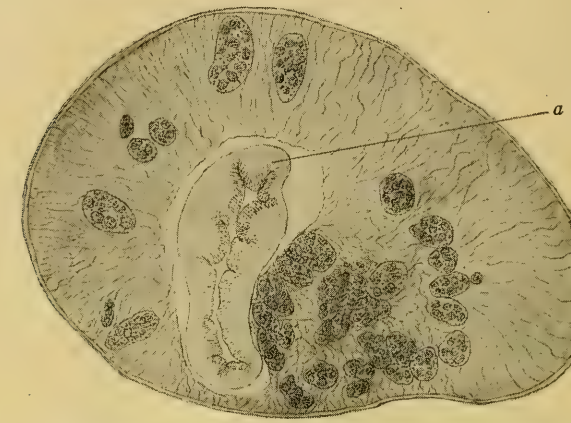

FIG. 5. Transverse section of *Hydroides dianthus* showing sporocysts embedded in the body wall. *a*, intestine.

There is considerable variation in the size of the sporocysts. The largest noted was 0.70 millimeter in length and 0.28 millimeter in diameter; the smallest 0.17 in length and 0.10 in diameter. In like manner the cercariæ varied in length, but the length of 0.12 millimeter for the anterior portion, and 0.36 for the tail, or 0.48 millimeter for the whole length, is not far from the usual length of a mature cercaria. One cercaria, living, had the following dimensions: Length of body 0.17 millimeter, breadth 0.04; length of tail 0.52, breadth 0.02.

What were interpreted to be striated muscle fibers were noticed in the tails of living cercariæ (Fig. 3). These fibers extend diagonally backward and inward from the exterior to the median line. They were about 0.0017 millimeter in diameter, and what appeared to be cross striations were plainly visible with a Zeiss D objective. Under an oil immersion lens their resemblance to striated muscle was evident.

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BIOLOGICAL BULLETIN

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NUCLEAR CHANGES IN THE REGENERATING SPINAL CORD OF THE TADPOLE OF RANA CLAMITANS.[1]

GEORGE FRED SUTHERLAND.

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I. STATEMENT OF THE PROBLEM.

The present paper gives the results of an histological study of the early stages of regeneration in the spinal cord of the frog tadpole, *Rana clamitans*. It deals especially with the degenerative nuclear changes immediately following the operation, and the phenomena of nuclear division in the formation of the new organ.

Fraisse (1885) studied these stages in several vertebrates in order to discover the origin of the regenerated tissues, and presented the following conclusions which may be used as a basis for a further detailed study.

"1. Sowohl bei Amphibien wie bei Reptilien sind verletzte Gewebe nur im Stande, wiederum gleichartig Gewebe zu erzeugen. Die Leukocyten übernehmen bei der Gewebsbildung nur die

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[1] Contribution from the Zoological Laboratory of the University of Illinois, No. 37.

Function der Ernährung; ausserdem nehmen sie zerfallende Gewebsproducte auf und assimiliren dieselben, um sie an anderen Orten wieder zu deponiren. Niemals werden sie selbst zu fixen Gewebszellen, weder in der Bindesubstanz noch sonst wo.

"2. Sämmtliche der in Frage kommenden Gewebe der Amphibien und Reptilien sind im Stande, sich zu regeneriren; entweder direct aus ihren Elementen, oder aus einer Matrix, so lange diese Matrix unverletzt ist. Als Matrix für die Epidermis ist das Rete Malpighii, für das centrale Nervensystem das Epithel des Centralcanales, für die Muskulatur die Muskelkörperchen zu betrachten.

"3. Zuerst regeneriren sich Epithel und Bindegewebe; beides scharf getrennt, ursprünglich aus gleichartigen Zellen bestehend, die sich später differenziren."

There remains the further problem of the stages in the process by which the old organs at the cut surface replace their lost parts. Two distinct kinds of changes take place in this process, (1) degenerative and (2) regenerative. First the injured cells at the cut edge degenerate. Then follows regeneration proper, or the formation of the new organ from the remaining elements of the old.

There are three ways in which regeneration proper might take place. (1) The cells at the cut edge of each organ by dividing might extend outward, and in time form the completed organ: (2) the cells in front of the cut edge might wander backward; and (3) the cells in front of the cut edge might divide in situ and push backward the more distal cells. These possible methods of regeneration will be made clearer by a diagram of that part of the hollow neural tube extending forward from the cut (Fig. 1). If (1) (division of cells at the cut edge) were the method of regeneration, we should find after the operation that the cells at the cut surface *A*, or from *A* to *C*, are dividing rapidly while from *C* to *B* about the normal number of cells is dividing.

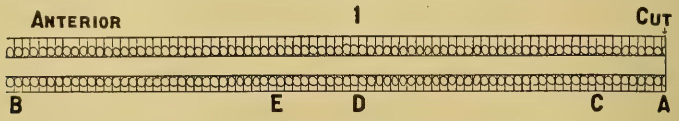

Fig. 1. Diagram, explained in the text.

If (2) (migration of cells) were the method, we might find no dividing cells at all, but should expect to find that the cells from *B* to *A* or possibly only from *D* to *A* are turned with their long axes parallel to the longitudinal axis of the spinal cord as if moving toward the cut end. If (3) (division of more anterior cells in situ) were the method, we should expect to find dividing cells all the way from *B* to *C* or possibly concentrated in a growing zone *ED*.

The present paper aims to give an account of the nuclear changes, both degenerative and regenerative, involved in the formation of the regenerated spinal cord.

II. Material and Methods.

Serial sections were made of tadpole tails killed after various regeneration periods. This enables one to follow the process from stage to stage. But to get uniform results from this method and eliminate individual variations, one must take tadpoles as nearly alike as possible at the start, operate on all at the same time, keep them under uniform laboratory conditions and make sections of several individuals at each stage.

On October 12, 1913, seventy tadpoles of *Rana clamitans*, varying in length from 30 to 60 mm., were brought into the laboratory. Two days later they were put into individual finger bowls, and forty-four medium sized individuals (32–40 mm. in length), chosen to constitute the main series, were grouped by twos or threes. Those of each group were as nearly alike as possible and each group was treated as a unit in the time of operation, killing, etc. The finger bowls were placed side by side on a table some distance from the windows so that uniform conditions of temperature, light, etc., were insured. None of the tadpoles was fed during the course of the experiment, and none died from the effects of laboratory conditions.

On October 15, the first operations were performed. Each tadpole was transferred from the finger bowl to a paraffin block and approximately one fourth of the tail was removed, with a sharp scalpel, at right angles to the plane of the tail. The animal was returned to the finger bowl and the removed part put into Gilson's killing fluid. At the end of the period of regeneration, the animals were again taken out onto the block and the regenerated tail plus a second fourth of the normal tail was removed and put immediately into Gilson's killing fluid. The times of killing were as follows: normal, immediately after the operation, 1, 3, 5½, 9½, and 14 hours, and 1, 2, 3, 4, 6, 8, 9, 10, 12, 14 and 16 days after the operation. Usual methods of technique were followed. Delafield's hæmatoxylin and acid fuchsin stain the nuclei blue and the cytoplasm pink, but do not distinctly bring out cell boundaries. For the most part sections were made in the sagittal plane.

III. Observations.

The study was confined to the histology of regeneration in the spinal cord, since a preliminary examination showed that this organ of all those in the tail was best adapted for a study of the present problem. Fig. 2 shows by a sagittal section the spinal cord, and its relation to the surrounding tissues. Fig. 3 shows a transverse section of the spinal cord alone. It is a hollow tube which distally is formed of a single layer of cells. The nuclei are very near the inner border of the cells so that there is a wide outer zone of cytoplasm but practically no inner cytoplasmic

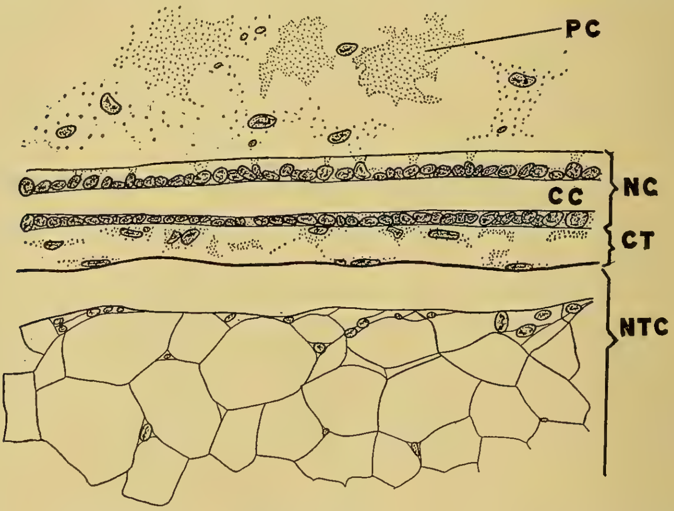

Fig. 2. Sagittal section through a part of the normal tail, showing the spinal cord and its relation to the surrounding tissues. *nc*, spinal cord; *cc*, central canal; *ntc*, notocord; *ct*, connective tissue; *pc*, pigment cell. (330 diameters.)

zone. At this stage in the development of the tadpole, the cells near the distal end of the spinal cord show little differentiation.

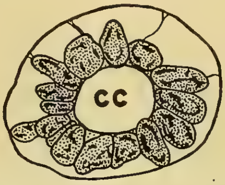

FIG. 3. Transverse section through the normal spinal cord, showing the nuclei and the outer cytoplasmic zone. *cc*, central canal. (890 diameters.)

I. *Degenerative Changes after an Operation.*

When a tadpole's tail is removed the old notocord extends out beyond the other tissues, and the connective tissue between the notocord and spinal cord is usually broken so that the spinal cord bends dorsally as in Figs. 7 and 8. A transverse cut through the tail leaves the various organs at the cut surface in contact with the surrounding medium, the water in which the tadpole lives. Sections of tadpoles killed immediately after the operation show the direct effect of the cutting (Figs. 4 and 5). Many nuclei and cells are broken and irregular in appearance and may be loosened or torn apart from each other. The injured nuclei at the cut edge and extending forward with decreasing frequency, are homogeneous in appearance and take a deep haematoxylin stain. Undoubtedly some of the nuclei are cut, and this accounts for the irregularity in shape of a good many. But a good many others, also staining deeply, are rounded and smaller than normal nuclei. These may be either normal nuclei which under the

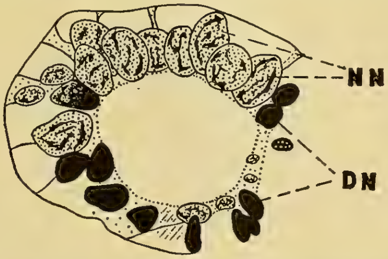

FIG. 4. Transverse section through the end of the spinal cord immediately after the operation, showing deeply-staining nuclei. *cc*, central canal; *nn*, normal nuclei; *dn*, deeply-staining nuclei. (920 diameters.)

stimulus of the operation are contracted or compressed, or cut nuclei which have rounded off. These deeply-staining nuclei, whether rounded or irregular in shape, are smaller than normal nuclei, so it may be that the chromatin, which stains deeply, is condensed on account of the loss of achromatic material.

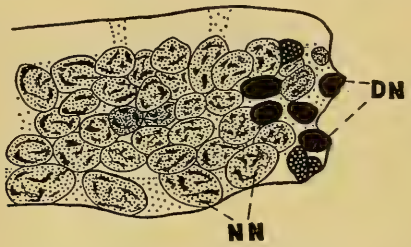

FIG. 5. Sagittal section through the side of the spinal cord immediately after the operation, showing the deeply-staining nuclei at the cut end. *dn*, deeply-staining nuclei. (920 diameters.)

The same assumption is borne out by the somewhat different appearance of nuclei in the tadpoles killed one hour after the operation (Fig. 6). Some are rounded as before; others are angular or slightly hour-glass shaped, with rather dense cytoplasm extending out from the corners. If parts of the nuclear membrane were held by the cytoplasm while the nucleus as a whole decreases in volume either by contraction or loss of achromatin, the nuclei might present such an appearance. Moreover there are gradations from hour-glass-shaped to normal nuclei

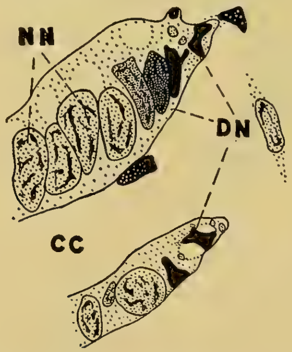

FIG. 6. Sagittal section through the spinal cord one hour after the operation. This shows the "contracting" nuclei. *cc*, central canal; *dn*, deeply-staining nuclei; *nn*, normal nuclei. (920 diameters.)

and corresponding gradations in size and depth of stain. In cases of this sort there are often vacuoles or cytoplasm between the nuclei as if the latter had shrunken, whereas in the normal cord, the nuclei are so close together that no cytoplasm can be seen between them. These facts indicate that normal nuclei become deeply staining nuclei by contraction or by loss of achromatic material.

This "contraction" of nuclei seems to be caused by contact with the water or killing fluid, or the succession of the two, as well as by direct injury from the scalpel, for other nuclei which are in contact with the exterior only through the central canal show this phenomenon. In some cases, the end of a nucleus nearest the central canal is deeply stained and contracted while the other part is normal (Fig. 4). The question immediately arises, why does not the water or other external factor enter the open neural tube and cause the contraction of the inner parts of practically all nuclei in the spinal cord? It is probably because of the presence in the tube of some substance which prevents the ready admission of external fluids, though capillarity would have a similar effect. Since the sections show very little structure within the central canal, this content must be liquid or semi-liquid. However, in a number of sections there is a rather long narrow band of cytoplasmic material which may be the more solid part of a semi-liquid substance coagulated by the killing reagent. There are other evidences of the presence of such a liquid. The sections from two of the tadpoles killed one hour after the operation show a coagulation of the outer surface of the blood plasma covering the wound, but over the spinal cord this coagulating process is delayed. The most plausible explanation seems to be that some cerebro-spinal fluid (compared by Barfurth to the cerebrospinal fluid of mammals) exerts an outward pressure which breaks through any slight hardening of the plasma at this point. Perhaps transference of the animal to a medium of different density, the killing fluid, aids the outburst. Sections of another tadpole killed at one hour show the presence of this coagulated plasma over the end of the spinal cord as well as over other parts of the tail.

The outward pressure of a fluid would tend to push out into the blood plasma any free elements such as the injured and degenerating nuclei with very little cytoplasm and hence little connection with other cells; and when this fluid breaks through, some of these nuclei may break off and float away. At one hour after the operation, broken and small rounded nuclei are seen in the end of the spinal cord and extending out into the hardened layer of the plasma, giving evidence of some force acting outward at this time (Fig. 7). Other evidences will be mentioned in describing the stages at which they appear.

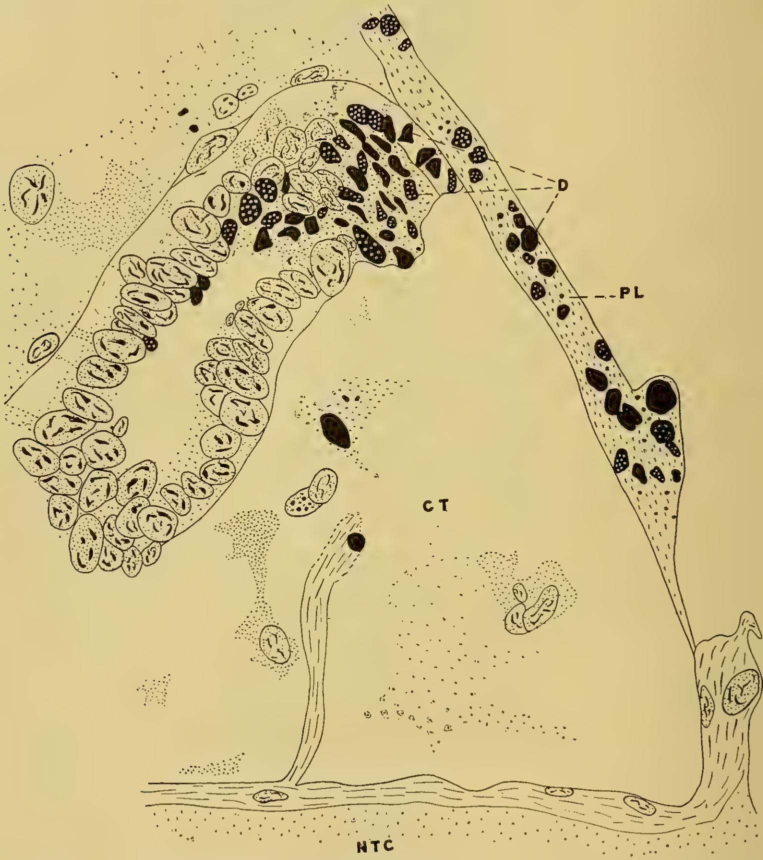

FIG. 7. Sagittal section through the spinal cord and the surrounding region one hour after the operation, showing irregularly shaped, deeply-staining nuclei in the end of the spinal cord and in the coagulated plasma layer. *dn*, deeply-staining nuclei; *pl*, plasma layer; *ct*, connective tissue; *ntc*, notocord. (1,100 diameters.)

Three hours after the operation there are fewer of the angular nuclei than at one hour and more of the round deeply-staining nuclei. The latter vary from the size of similar ones in the earlier stages down to fragments. Moreover some of the larger of these seem to be in the process of fragmentation, that is, appearances indicating stages in direct division are seen. The gradation in size and depth of stain at one hour from normal nuclei nearly to rounded ones, and the gradation down to fragments at three hours, as well as the appearances of fragmentation, make it fairly clear that normal nuclei just in front of the cut edge may contract, become rounded, and fragment. This must be a degenerative process. Even finer intermediate steps are seen in preparations of later stages.

Sections of one individual at this period appear very much like those immediately after the operation. The deeply-stained nuclei are similar, and the spinal cord is not covered either by epidermis or plasma, so that a recent outbreak of the cerebrospinal fluid must have taken place. In this case a second contact with the exterior has again started the degenerative process.

At five and a half hours the spinal cord is entirely covered by the thickened plasma layer, in which is a group of fragmenting globular nuclei. In one preparation at this time, the epidermis has closed-in over the entire wound, and there is a series of stages in the degeneration of nuclei. Some are only slightly smaller and darker than normal nuclei; others have the angular appearance characteristic of nuclei one hour after the operation, while still others are round and fragmenting. At this stage there is another evidence of the presence of a cerebrospinal fluid. The plasma covering the end of the spinal cord is pushed outward, making a knob-like extension of the central canal similar to that shown in Fig. 8. This did not appear in earlier stages either because not enough cerebrospinal fluid was present, or because the plasma layer had not coagulated sufficiently to resist the outward pressure of this fluid.

Of the two preparations of tadpoles killed after a nine and a half hour interval, one shows the epidermis and plasma covering all the wound except the neural tube; the other shows this part also covered. In the former, the sides of the neural tube are separated as if by a recent outburst of cerebrospinal fluid, and deeply-staining rounded and fragmenting nuclei are seen. In the second preparation, the deeply-staining nuclei are all small and fragmentary. In other words no more nuclei seem to be starting to degenerate.

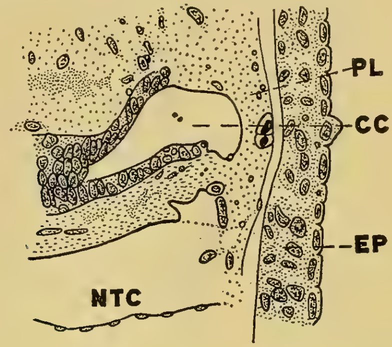

FIG. 8. Sagittal section through the end of the spinal cord fourteen hours after the operation. This shows the epidermal layer, the plasma layer, and the knob-like extension of the central canal, caused by the outward pressure of the cerebrospinal fluid. *ep*, epidermis; *cc*, central canal; *pl*, plasma layer; *ntc*, notocord. (330 diameters.)

At fourteen hours, plasma and epidermis cover the spinal cord though the plasma is pushed outward by the cerebrospinal fluid (Fig. 8). There are nuclear fragments in the cord and degenerating nuclei in the plasma. Another preparation of the same period shows the nerve cord still open to the exterior, as well as the nuclear appearance of an earlier stage.

At twenty-four hours, only a few of the nuclei are slightly smaller and darker than the normal. At this time there appear near the end of the spinal cord, granular leucocytes containing pigment granules and fragments which closely resemble the fragments of degenerating nuclei. It may be that the leucocytes appear at this time and dispose of nuclear fragments. After one day, the degenerating nuclei are too rare to be significant.

The degenerative process which the foregoing facts seem to show, may be indicated diagrammatically as follows:

Cells directly cut → broken nuclei → rounded nuclei → fragments → disposed of by outbreak of cerebro-spinal fluid, or by leucocytes.

Cells just in front of those cut → angular nuclei → rounded nuclei → fragments → disposed of by leucocytes.

2. *Enlargement of Nuclei.*

A few preparations of the spinal cord soon after the operation show plainly that the nuclei near the end, but just in front of the deeply-staining nuclei, are larger than those of the normal cord. The long axes of nuclei close to the edge were measured and compared to nuclei of the same preparation which are some distance forward in the old tissue (Table I.). Immediately after the operation and in the very early regeneration stages, the nuclei near the end are larger, but the difference decreases until after nine and a half hours it is hardly significant. This enlargement might be preparatory to normal division or it might be a swelling which is a degenerative change preliminary to fragmentation Since this size difference is greatest at the very beginning and decreases during the first day until it is no longer significant, and since mitotic divisions are not seen in numbers until the third day, the enlargement is probably an early stage in nuclear degeneration.

TABLE I.

Time of Regeneration.	Nuclear Length Close to Edge.	Nuclear Length in Front of Edge.	Difference in Length.
Normal	7.9	7.5	.4
Immediately	10.5	7.8	2.7
1 hour	11.3	9.1	2.2
3 hours	8.6	7.3	1.3
5.5 hours	12.8	10.5	2.3
9.5 hours	8.1	8.0	.1
14 hours	7.6	8.3	−.7
1 day	8.2	6.6	1.6
2 days	8.1	8.0	.1
3 days	8.2	8.9	−.7
4 days	8.4	8.2	.2
6 days	10.7	10.4	.3

Explanation.—Each measurement recorded here is the average of the measurements of 9 or 10 nuclei. These were recorded in terms of the spaces of the ocular micrometer, but since one space was equal to approximately one micron (.955), the measurements were not transposed.

3. *Temporary Partial Closing of the Spinal Cord.*

After the degenerative process is complete and the deeply-staining nuclei have disappeared, the end of the nerve cord starts to close over. By the first day, the nuclei in the end of the cord have begun to pull apart, stretching out the connecting cytoplasm (Fig. 11). In general they extend toward the opposite

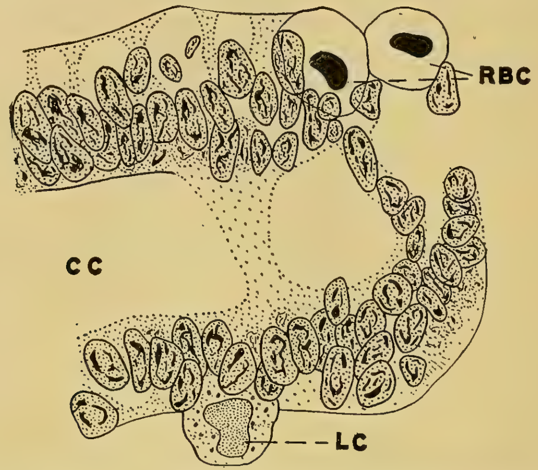

FIG. 9. Sagittal section close to the edge of the central canal, showing a row of cells, not quite at the end, extending across the central canal. Other sections of the series show that the end of the cord is still open. *cc*, central canal; *rbc*, red blood corpuscles; *lc*, leucocyte. (920 diameters.)

wall of the central canal, thus narrowing the opening at the end. Some sections show pseudopod-like cytoplasmic extensions of the cells into the central canal as if closing were to be produced

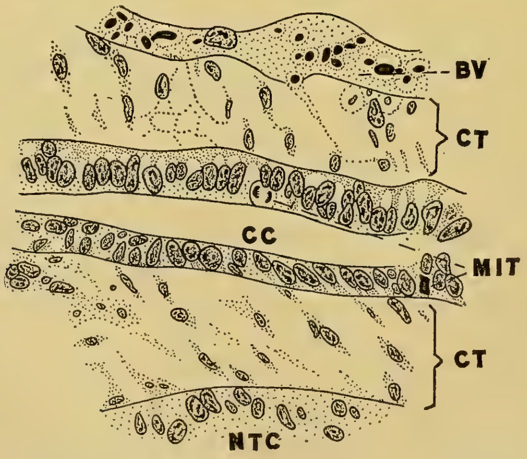

FIG. 10. Sagittal section through the new spinal cord six days after the operation. *bv*, blood vessel; *mit*, mitotic figures; *ntc*, notocord; *ct*, connective tissue. (330 diameters.)

by amoeboid movement of the cells. Figure 9 shows a section through one side of the cord, in which one layer of cells, not quite at the end, is extending down into the central canal. Up to about six days phenomena such as these may be seen, but sections from six to sixteen days show that the closing is not completed within that period. By sixteen days the new tail is

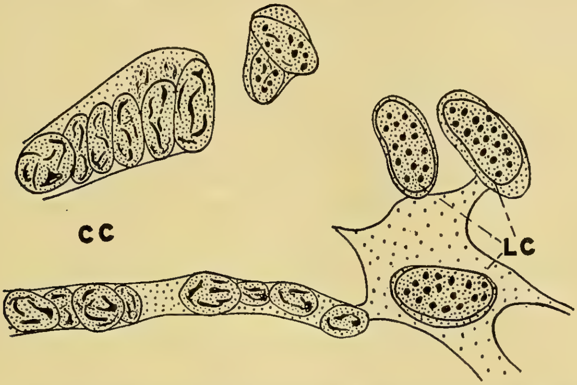

FIG. 11. Sagittal section through the spinal cord one day after the operation showing the granular leucocytes at the end of the cord, and the pulling apart of nuclei in the lower part of the cord. *cc*, central canal; *lc*, leucocytes. (920 diameters.)

almost as long as it will become (Durbin, 1909), and the spinal cord reaches back close to the epidermis at the posterior end. Still these later preparations show the sides of the neural tube gaping open, and red blood corpuscles extending forward into the central canal of the new cord, as if the pressure of the cerebrospinal fluid is not sufficient to keep them out.

4. *Cell Division.*

In an organ such as the spinal cord in which the nuclei lie close together, it is difficult to determine an amitotic division. In order to be sure that amitotic divisions do occur, one must find continuous stages in nuclear and cellular constriction without the formation of chromosomes. Because of the massing of nuclei, this cannot readily be determined in the normal spinal cord, though the slides were examined with this point in mind. The present study gives no evidence that normal nuclei divide amitotically, but stages in direct division can be seen in the deeply-staining nuclei at the cut edge. Is this amitosis or fragmentation? Do the daughter nuclei form normal nuclei, or do they divide several times and degenerate? There is no definite evidence that nuclei which divide directly ever become normal again. But at successive stages the deeply-staining nuclei become smaller and smaller down to fragments, so that the direct division is probably a fragmentation as a part of the degeneration of injured nuclei.

Mitotic divisions can easily be distinguished by the formation of chromosomes. All the preparations were examined and the distance of each mitotic division from the cut edge was measured. The results are shown in Table II. In the sections of the normal tail the number of divisions is the smallest, but since up to three days the mitoses are scattered and the number of individuals small, there is no reason for considering these mitoses anything but normal. During the period of degenerating nuclei, there are almost no mitotic divisions close to the edge. On the third day, the nuclei just in front of the cut edge are proliferating rapidly; at four days there are a few divisions past the cut; at six days there are almost as many divisions in the new spinal cord as in the old; at eight and nine days most of the divisions are in the new cord; at fourteen days there are scattered mitoses only, both in the old and new cord, and at sixteen days most of the dividing cells are in the very end of the new cord. If later divisions follow this general trend, it seems likely that the rest of the spinal cord will be formed by a growing zone at the tip, and until the new cord is complete the number of mitoses near the tip would probably decrease gradually.

Fig. 12 gives the average number of mitoses in the spinal cord at each stage, and therefore represents the rate of growth at these times. On the second day there is a considerable mass of tissue over the whole wound, though only degenerative changes have been taking place in the nerve cord. Beginning about this time, the nuclei in the end of the cord loosen and draw apart somewhat, stretching out the cytoplasm between them (Fig. 11). This is apparently the first extension in length of the spinal cord. At three days active proliferation of cells has begun but the pulling apart or stretching toward the cut edge continues. Fig.

TABLE II.

Regeneration Time.	No. of Individuals.	Mitoses in Old Cord.	Mitoses in New Cord.	2.8 mm.	2.1 mm.	1.4 mm.	.7 mm. ← Old Tissue.	Position of Cut. New Tissue. →	.7 mm.	1.4 mm.
Normal . .	3	⅔	0		⅓	⅓				
Immed. . .	3	2⅔	0	⅓	⅓	⅓ ⅓ ⅔	⅓ ⅓			
1 hr.	3	6	0	⅔ ⅔	⅔ 1 ⅓	⅓ ⅓ ⅔	⅓ ⅓ ⅔			
3 hrs.	2	1½	0	1	½					
5½ hrs. . . .	2	2½	0	1 ½		½	½			
9½ hrs. . . .	2	1½	0		½ ½	½				
14 hrs. . . .	2	3½	0	½	½ ½	1 ½	½			
1 day. . . .	3	1⅓	0		⅓	⅓	⅓ ⅓			
2 days. . .	4	1⅓	0		⅓	⅓	⅓ ⅓			
3 days. . .	1	19	0				6 4 8 1			
4 days. . .	2	15	2½	½ ½ 1	½	½	½ 3 2½ 6	1 1½		
6 days. . .	2	19½	19	½ ½ ½		1 1 ½ 2	2½ 2 2½ 4 2½	4 3½ 2½ 6½ 2½		
8 days. . .	1	2	40				1 1	3 12 6 5 4	1 3 6	
9 days. . .	1	16	42			1 1 1 3	3 1 6	2 7 4 5	3 3 4	9 5
14 days. .	2	6	6		½	½ ½ 1½	1½ 1 ½	1 1 ½ 1	½ 1 1	
16 days. .	2	½	7½			½		1 ½		3 3

This table represents the position and number of mitotic divisions taking place at different times. In most cases the number given is the average obtained from 2 to 4 individuals, as indicated in the table.

10 (six days' regeneration) shows the cells in one part of the cord stretched out to such an extent that vacuoles are left between the cells. It is during the period from four to sixteen days that most of the increase in length takes place, by active proliferation and migration of cells.

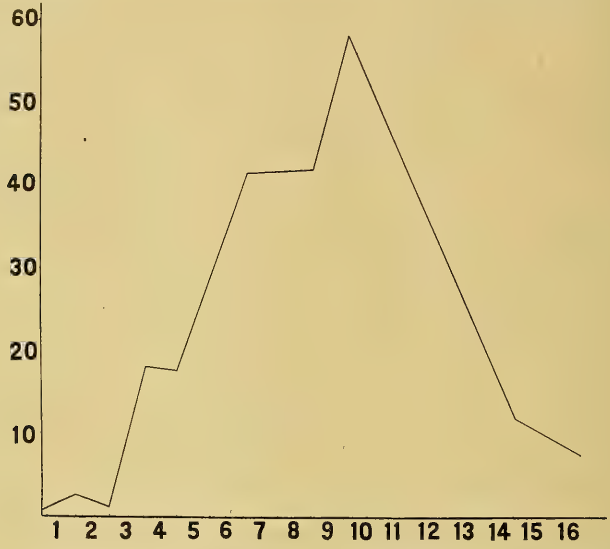

FIG. 12. Curve giving the number of mitotic divisions in that part of the spinal cord within 3 mm. of the edge. Beyond 3 mm. the mitoses are scattered. An abscissa represents the period of regeneration and the corresponding ordinate gives the average number of mitoses found in the individuals killed at the end of that period.

IV. Discussion.

1. *Amitosis and Fragmentation.*

Fraisse in describing the regulative process at about two days after the operation, says; "Bereits früher machte ich darauf aufmerksam, dass am Wundrande eine starke Auswanderung von Leukocyten stattfindet, und dass diese es sind, welchen vor allen Dingen die Bildung des homogenen, lymphartigen Saumes, welcher zuerst die wunde bedeckt, zuzuschreiben ist. Das Rückenmark geht nun an meinen Schnitten bis dichte an diesen homogenen Saum heran, und die Elemente, welche es zusammensetzen, lassen sich immerhin noch nach 24 Stunden auch an diesem Saum von einander trennen, dann aber tritt eine bedeutende Wucherung von Kernen auf, und zwar scheint dieselbe auszugehen von den sogenannten Körnern,[1] deren Inhalt völlig homogen und stark lichtbrechend erscheint. Durch Picrocarmin werden diese Elemente ebenfalls stark tingirt, und nun sieht man an diesen nahezu gleich grossen Körnern Kerntheilungen, ohne dass jemals eine Spur von karyokinetischen Figuren constatirt werden konnte, in der Weise auftreten, das der Kern oder die Körner sich in der bekannten Weise schuhsohlenförmig einschnüren, und dass dann aus beiden Hälften Elemente gleicher Art hervorgehen. Nicht nur eine einmalige Einschnürung glaube ich beobachten zu können, sondern auch eine mehrfache, so dass der Kern sich bei diesem Process nicht nur in zwei, sondern auch in mehrere Stücke theilen kann."

Fraisse discusses further the evidence that the nuclei from the end of the spinal cord, which are found in the lymph-like border, divide amitotically. This agrees with the present observations. But he is satisfied to show that direct division does take place. So far as my preparations show, there are few evidences that the nuclei which divide amitotically afterward become normal nuclei. In some of the preparations of stages at which the deeply-staining nuclei have almost disappeared, there are a few nuclei which stain only slightly darker than the normal ones, and at this time there are no stages between these and the fragments. These few slightly darkened nuclei may, then, be forming normal nuclei again. All other evidence points towards the conclusion that at successive stages, these deeply-staining nuclei become smaller and smaller as if fragmentation or repeated direct division, is taking place. The conclusion from these facts is that nuclei which have only started to degenerate may perhaps return to the normal condition, but that nuclei that have gone so far as to divide amitotically are destined to fragment.

2. *The Appearance of Leucocytes.*

Barfurth (1891), working on the regenerating spinal cord of the frog larva at forty-six hours and at three days, makes the following statement: "Die unterste Theil des regenerirten Medullarrohres beherbergt in seinem Innern und zwischen seinen Epithelzellen zahlreiche fettig degenerirende Leukocyten; viele kleine und grosse Fetttropfen, die man hier überall findet, führe ich ihrem Ursprunge nach auf solche zerfallene Wanderzellen zurück. Ausserdem finden sich hier auch viele Pigmentkörnchen, die wohl bei der regressiven Metamorphose der zerfallenden Leukocyten entstehen (Pigmententartung)."

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[1] Körnern-nuclei of the gray substance, which are not present in the distal region of the spinal cord.

Barfurth figures the spinal cord of a larva of *Triton cristatus* after the sixth day of regeneration, in which these leucocytes and fat drops are shown. His figure is very similar to Fig. 11, which shows a section of a tadpole killed twenty-four hours after the operation. Both Fraisse and Barfurth mention particularly the presence of leucocytes in the early regeneration stages, but in the present study, leucocytes were not found in large numbers. Up to the end of the first day, none at all were seen close to the spinal cord. The earliest stage mentioned by Barfurth is that after a forty-six hour regeneration period, and this probably accounts for the different interpretation he gives of the origin of the "Fetttropfen" or fragments. If these fragments are followed back into earlier stages in my sections, they become larger and larger and are seen to be identical with the degenerating nuclei. To be sure, the leucocytes when they first appear in the spinal cord region contain what might be called fat drops, but is it not more reasonable to suppose that the leucocytes which are present at this time dispose of the fragments of injured spinal cord nuclei?

3. *Temporary Closing of the Spinal Cord.*

Barfurth describes the closing of the spinal cord at three days by means of cytoplasmic extensions of the cells, such as were seen in the preparations used in the present study. "Der sich wieder ansammelnde Liquor cerebrospinalis drückt nun auf die neugebildeten, noch wenig resistenten untern und seitlichen Theile des Rohres, und treibt sie kolbenartig auseinander. Die Zellen passen sich einstweilen durch ihre Lagerung diesem Druck an und behalten später diese Lage noch eine Zeit lang bei." Barfurth mentions this as a temporary closure of the spinal cord, so his later preparations evidently show the cord again open. The regenerated spinal cord at sixteen days has almost reached its maximum length, but it is not yet closed. Whether or not the completely regenerated spinal cord is open at the end or closed as in the normal tail cannot be answered by the present study.

4. *Rate of Division. Amitosis versus Mitosis.*

Durbin (1909), in analyzing the rate of increase in length throughout the regenerative process in the tail of *Rana clamitans*, distinguishes four periods. "The operation was followed by an interval of low rate, succeeded by one of rapidly increasing rate, then by one of rapidly decreasing rate and finally an interval in which the rate gradually approaches zero. The first low period is explained by a combination of two factors, (*a*) the shock of the injury, and (*b*) the formation of a cap of embryonic cells which is to serve as a basis for the more active regeneration. The second or period of rapidly increasing growth is the one in which practically all the cells in the new part are undifferentiated and rapidly dividing. The third and fourth periods are explained by the appearance of differentiation, which lessens the number of dividing cells."

Fig. 12, based on the number of mitotic divisions in the spinal cord, shows these same periods. The initial period of low rate covers the first two days; that of rapidly increasing rate includes the third to ninth days; the period of rapidly decreasing rate extends from the tenth to sixteenth days, and the period of gradually decreasing rate, though not covered in the present work, would undoubtedly extend on from about sixteen days. In the light of this histological study, a somewhat different interpretation might be given to the initial period. It is during these first two days that degeneration of the injured cells is taking place. Though at this time a cap of undifferentiated cells is being formed over the wound, the spinal cord does not participate in the formation of this cap, nor is any such cap formed at the end of the spinal cord. Since the spinal cord cells in this part of the tail are so slightly differentiated, the new cord is formed from the old without the separation of a group of special embryonic cells.

The similarity of the rate curves based on a counting of the mitotic divisions with that based on the amount of tissue formed at each period, seems to be significant. It shows that the rate of tissue formation is closely correlated with the number of mitotic divisions. Considering amitosis, this may be interpreted in one of two ways—(1) either the number of amitotic divisions is similarly correlated with the rate of growth so that the total number of divisions both mitotic and amitotic, gives the same form of curve as the mitotic divisions alone, or else (2) amitotic divisions are not numerous enough to be significant. The former explanation is improbable. The nuclear conditions producing mitotic division are probably different from those producing amitotic division. Different cells in the same region may divide by different methods, but it is very improbable that the conditions producing one form of division would increase and decrease in influence at the same rate and the same times as those producing the other form. Moreover, in the present study, no examples of direct division were seen except in the degenerating, fragmenting nuclei. This similarity of the rate curve of mitotic divisions to the rate curve of growth is evidence, other than the negative observational evidence, supporting the view that amitotic division is not important in the formation of this organ by regeneration.

V. SUMMARY.

1. The regenerating spinal cord of the frog tadpole has been studied histologically in order to learn the mechanism, or the stages in the process, by which the new cord is formed from the old.

2. During the first day after the operation, injured nuclei in the end of the spinal cord degenerate. There is first a decrease in size, by contraction or loss of achromatin, and then a fragmentation of these degenerating nuclei. The fragments may be carried away either by the outbreaking of a cerebrospinal fluid or by leucocytes which appear at this time. These fragments are parts of disintegrated spinal cord nuclei and not of leucocytes.

3. From the second to the sixth days there is a temporary partial closing of the neural tube, probably by migration of the cells near the end.

4. The new cord is formed by the cells of the old cord near the cut edge, by mitotic division and migration.

5. The number of mitotic divisions at different periods is proportional to the rate of regeneration at those periods as determined by Durbin. Amitotic division, if it occurs, is not important in the formation of the regenerated organ.

6. There is no observational evidence from this study that amitotic division does occur in normal regenerating spinal cord cells.

This work was carried on under the direction of Dr. Charles Zeleny. His suggestion of the problem, and constant interest in its progress are sincerely appreciated.

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NOTE ON THE EFFECT OF X-RADIATION ON FERTILIZIN.[1]

A. RICHARDS AND A. E. WOODWARD.

The observations of one of the writers that x-rays would produce changes in the activity of certain enzymes suggested that these rays might perhaps be effective in bringing about changes in the action of cell extractives, particularly of fertilizin, the descriptions of which by Lillie and Glaser appeared at the beginning of the summer. The opportunity was presented to test this suggestion during the past summer at Woods Hole, since the one of us carried on studies on the effect of x-rays on some marine eggs and the other continued the work on fertilizin of *Arbacia* begun by Glaser. This note gives a summary of the results. It is realized by the writers that the study is by no means a complete one, but it is believed by them that publication is justified in view of the facts that the experiments give clear evidence on the main point under investigation and that there is at present no prospect of opportunity for their further work on the problem.

In taking up these experiments the writers felt that if it should be shown that x-radiation influences the activity of the cell extractive called fertilizin, that fact would be of interest from several view points: (1) without regard to the nature of fertilizin or its rôle in the fertilization of the egg, it is a substance derived from the eggs which has the property of being definitely modified by those external agents of which experimental use may be made; (2) in cell extractives, of which fertilizin is an example, there is a basis for the action of x-rays upon living cells, and doubtless the marked effects of the rays upon tissues is partially due to such action; (3) the modifiability of its activity by radiation is an interesting property of fertilizin; (4) this property may serve as a point in determining the relation of fertilizin to enzymes.

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[1] Contribution from the Marine Biological Laboratory at Woods Hole, and from the Zoology departments of the University of Texas (No. 123) and of the University of Michigan.

The methods used in these experiments were largely those of Lillie and Glaser. The solution used as a standard was prepared according to the method of Lillie by "adding to a certain number of 'dry' eggs, double their volume of sea-water, and with occasional slight agitation allowing ten minutes to elapse. At the end of this time the ova were precipitated by 100 revolutions of the centrifuge and the supernatant fluid, a clear, golden liquid in the case of *Arbacia*," was decanted, (Glaser, 14*a*). The agglutination of fresh sperm in suspension by fertilizin in both control and radiated solutions, was tested by the unit concentration method of Lillie, of which he says, (13*b*) "The agglutination reaction of the sperm in the presence of this substance (*Arbacia* fertilizin) is, as noted in previous studies, reversible, and the intensity and duration of the reaction is a factor of the concentration of the substance. The entire reaction is so characteristic that it was possible to arrive at a unit by noting the dilution at which the least unmistakable reaction was given. This was fixed at about a five or six-second reaction, which is counted from the time that agglutination becomes visible under a magnification of about 40 diameters until its complete reversal. The unit is so chosen that a half dilution gives no agglutination of a fresh 1 per cent sperm suspension." Further details are given in his recent paper ('14, pp. 526–528). One can best observe the details of the reaction with the low power of the microscope. The sperm suspension is mounted under a cover glass and the drop of fertilizin added at the edge of the suspension by means of a pipette. The entire process is observed through the microscope, and the time elapsing before the complete reversal of the reaction is carefully noted by means of a stop-watch. Thus it was possible to determine the degree of activity of a given sample of fertilizin, and by comparing radiated and non-radiated solutions, to measure the effect of the radiation by x-rays.

Another possible method of studying the effect is suggested by the fact that fertilizin can be used to bring about the parthenogenetic development of *Arbacia* eggs, the so-called auto-parthenogenesis. The efficacy of fertilizin before and after radiation in bringing about auto-parthenogenesis is a measure of the action of the radiation on it.

In all the experiments with sperm it has been our policy to use only data from clear cut reactions in which the beginning and the end of the agglutination were definitely marked. Precautions were taken to see that the sperm suspension was fresh and clean. Lillie has shown that both of these factors are important, for an old suspension becomes inactive and the presence of impurities such as blood acts as an inhibitor of the reaction.

Previous experience (Richards, 14) has shown the radiations to be of three kinds in relation to their effect on enzymes depending on duration, intensity and distance of the object from the x-ray tube; namely, accelerative, non-effective, and inhibitive. Under the conditions which usually prevailed in these experiments, a short exposure, of about 2 minutes, is accelerative; an exposure of about five minutes is non-effective; and one of longer duration becomes inhibitive. In view of these facts, similar exposures of fertilizin were made and the resulting activity tested as already explained.

In a preliminary experiment on July 8 the following figures were obtained as the average of a number of readings of the time elapsing before the complete reversal of the agglutination reaction after short and long radiation of fertilizin. The fertilizin solution used was about 2 per cent. standard strength (in this early experiment the strength was not accurately determined, but it is not strictly necessary under the conditions of this test that it should be known exactly). For the control, non-radiated solution the average reaction time was 32 seconds; for the 2-minute radiation the average time was 33 seconds; and for the 15-minute minute radiation it was 23½ seconds. This solution was then diluted to one-half and these figures obtained: Control, 19 seconds; 2-min. radiation, 20 seconds; 15-min. radiation, 16 seconds. This experiment is incomplete and the differences lie nearly within the limits of variation, but they suggest definitely that the short radiation rendered the fertilizin slightly more active (that is, enabled it to hold the sperm in agglutination longer), and the long radiation caused it to be less active than the control. More decisive data would have been given had the dilutions been continued to unit concentration, a fact which led to the adoption of that method in subsequent experiments.

In another experiment (July 14) a 1/50 dilution (2 per cent. standard) of *Arbacia* fertilizin was used. It was separated into four parts, of which one (Sc) was kept as a control solution, one (S2) was radiated 2 minutes, one (S5) five minutes, and the last (S7) seven and a half minutes. The results of these solutions when tested for their agglutination time at successive dilutions to unit concentration are given in the following table. ½Sc means control solution diluted to one-half; ¼ Sc, diluted to one-fourth, etc. The difference between two successive reaction times is marked *d*. Unit concentration is indicated by the asterisk(*).

TABLE I.

Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.
Sc.	34 sec.		S2	37 sec.		S5	34 sec.		S7	29 sec.	
$\frac{1}{2}$ Sc.	22 sec.	12	$\frac{1}{2}$ S2	23 sec.	14	$\frac{1}{2}$ S5	27 sec.	7	$\frac{1}{2}$ S7	22 sec.	7
$\frac{1}{4}$ Sc.	17 sec.	5	$\frac{1}{4}$ S2	15 sec.	8	$\frac{1}{4}$ S5	19 sec.	8	$\frac{1}{4}$ S7	17 sec.	5
$\frac{1}{8}$ Sc.	10 sec.	7	$\frac{1}{8}$ S2	11 sec.	4	$\frac{1}{8}$ S5	12 sec.	7	$\frac{1}{8}$ S7	12 sec.	5
$\frac{1}{16}$ Sc.	4–5 sec.*	5	$\frac{1}{16}$ S2	7 sec.	4	$\frac{1}{16}$ S5	5 sec.*	7	$\frac{1}{16}$ S7	8 sec.*	4
			$\frac{1}{32}$ S2	4 sec.*	3				$\frac{1}{32}$ S7	0 sec.	

Inspection of this table shows that the activity of S2 was increased by the short radiation, for five dilutions were required to reduce it to unit concentration, whereas that state was reached in four dilutions in the other three solutions; also the full strength of this solution held the sperm in agglutination longer than did that of the control, 37 against 34 seconds. In other words, Sc was 800 units agglutinating strength, S2 was 1,600 units, S5 and S7 were each a little over 800 units, and much below 1,600 units strength. (Lillie, '14, p. 527.)

The number of dilutions required in S5 was the same as in Sc and the sperm were agglutinated the same time by both solutions. This is in line with the previous experience that a radiation of about five minutes' duration under the conditions of these experiments is non-effective. However, these figures give an additional fact of possible significance which has not been entirely confirmed by other experiments either on fertilizin or on enzymes such as pepsin. If *d* represents the differences between the number of seconds required for the reversal of the reaction by successive dilutions, its value in S5 is practically a constant, 7; but in Sc and

S2 it begins as a large number and decreases rapidly: in Sc its successive values are 12, 5, 7 and 5, while for S2 they are 14, 8, 4 and 4. In S7 the values of *d* are smaller and decrease more slowly, being 7, 5, 5 and 4. This suggests that the laws governing the agglutination reactions by the various solutions are of different character. But in as much as this interesting result has not been generally obtained it is not possible to attach special importance to it at this time. It is given merely as suggestive.

The data in the case of S7 indicate that the activity of the fertilizin was decreased although the number of dilutions was the same as in the control, because the number of seconds required for the reversal of the reaction at unit concentration was much larger than is usual; yet at a further dilution no reaction was obtained. Also the undiluted solution did not hold the sperm in agglutination as long as in the control. Furthermore, it may be significant that the value of *d* for S7, as indicated above, are smaller than in the case of the other solutions.

Subsequent experiments along the same line gave similar results. They show clearly that radiation by x-rays is capable of changing the activity of fertilizin, and in general agree with previous work that weak radiation is accelerative and strong inhibitive. Some of our experiments were performed during the latter part of the summer at the end of the breeding season and there were irregularities in the results, but it is believed that these irregularities may be attributed to the unsatisfactory condition of both sperm and eggs at this season of the year and that the statement above gives the true effect of radiation on fertilizin.

Also during the latter part of the summer the writers tested the effect of x-radiation on fertilizin with regard to its power of inducing auto-parthenogenesis. Due to the near end of the breeding season these results are not entirely trustworthy, but they agree fully on one point, namely, that the radiation effects changes in the capacity of fertilizin to induce parthenogenesis.

On August 10 a sperm agglutination experiment was performed which possibly throws some light on the irregularity of the auto-parthenogenesis and at the same time makes the auto-parthenogenesis test doubtfully applicable for the radiation problem. This experiment gave data showing that the radiation effects wore off when the fertilizin had stood for some time. If this is true in general it must follow that, since the fertilizin must stand in the parthenogenesis experiments, there would be irregularity in the results.

The only tests of the effect of x-radiation on *Asterias* fertilizin were made on July 28, when the fertilizin was divided into four portions, as usual. One was kept for a control, one radiated two minutes, one five minutes, and the fourth fifteen minutes. The fertilizin was then put on mature *Asterias* eggs, which were allowed to stand two hours in the solution. They were then rinsed with sea-water and treated with hypertonic sea-water (50 c.c. sea water + 8 c.c. 2.5 M. NaCl) for thirty minutes, washed again with sea water, and allowed to stand for 12 hours. All four lots of eggs showed parthenogenetic development, and those treated with fertilizin which had been radiated 2 minutes had a much larger percentage of cleavages than either the control or the others.

Several times *Arbacia* fertilizin was similarly subjected to x-rays and then tested for its auto-parthenogenetic effect on fresh *Arbacia* eggs. The experiments are not satisfactory, because in most cases eggs from the same females gave abnormal results when tested in other ways. The following summarizes the more interesting experiments. Percentages were obtained by counting about 200 eggs.

TABLE II.

	Experiment I.		Experiment II.		Experiment III.		Experiment IV.		Experiment V.	
	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.
Sperm control			23.8		46.6					
Fertilizin control (unradiated)	30.2	0	9.5	0	5.2	1	20.2	.5	15.5	
Fertilizin 2 min. radiation.....	24.3	few	14.1	1	10.8	3	13.5	0	28.5	2
Fertilizin 5 min. radiation	21.6	"	17.9	0	8.4	0	10.5	0	87.2	
Fertilizin 15 min. radiation ...	17.5	0	15.6	.5	9.5	0	13.6	0	52.1	

Since the effect of x-radiation on fertilizin seems to be similar to its effect on enzymes, it is of interest to note the fact that the efficiency of the agglutinin contained in fertilizin, like pepsin (Euler, p. 132) varies with the square root of the concentration. If the efficiency is measured by the number of seconds the sperm remain agglutinated, and the concentration is measured by units of strength, the curves in Figs. 1, 2, and 3 are obtained for the readings of July 14, August 10, and August 11, respectively. The average is shown in the dotted line of Fig. 4. If an equation is worked out for this curve, we obtain $y^2 = 11x$ where y represents the efficiency and x the concentration. This equation is plotted as a solid line on Fig. 4. In the higher dilutions, of which a greater number of values were averaged, and where readings could be made more accurately, the curves coincide very closely. In the less dilute portion the coincidence is not so marked, but is still within the limits of experimental error.

The writers are not now able to offer an opinion as to whether or not fertilizin has the character of an enzyme. The coincidence, however, in the behavior of this substance, when treated by x-rays, to that of true enzymes, is indeed striking.

While the nature and composition of fertilizin are as yet unknown, it is a cell-extractive which is capable of undergoing changes under the action of experimental agents such as radiation by x-rays. Possibly it, or its forerunner, exists in the egg in combination. Among the other constituents of *Arbacia* eggs, this substance stands as one which, at least in solution in sea water, is able to bring about certain reactions on the part of sperm, and these reactions are subject to experimental modification. This justifies the inference that this substance or perhaps some similar one within the egg may be capable of undergoing modification in its relations to the various intra-cellular activities.

In this modification we may look for the seat of part of the changes which are brought about in living tissues and especially egg cells by radiation. The Hertwigs, Packard and others have shown that the chromatin of such cells is affected, and there is good evidence that the cytoplasm as well is influenced. Changes in their activity have also been demonstrated in the case of enzymes. These experiments add still another to the list of substances which are affected by the action of x-rays. It is probable that fertilizin is simply one example of a group of substances which may be the object of such action (but an example which may be studied). It is to be noted that these experiments render untenable the conclusion of the Hertwigs, that chromatin is the chief and perhaps exclusive seat of the effects of radiation upon eggs. Fertilizin is a substance doubtless without morphological representation in the structure of the egg; yet it may suffer considerable modification from x-ray treatment.

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FIGURE 1 – (JULY 14)

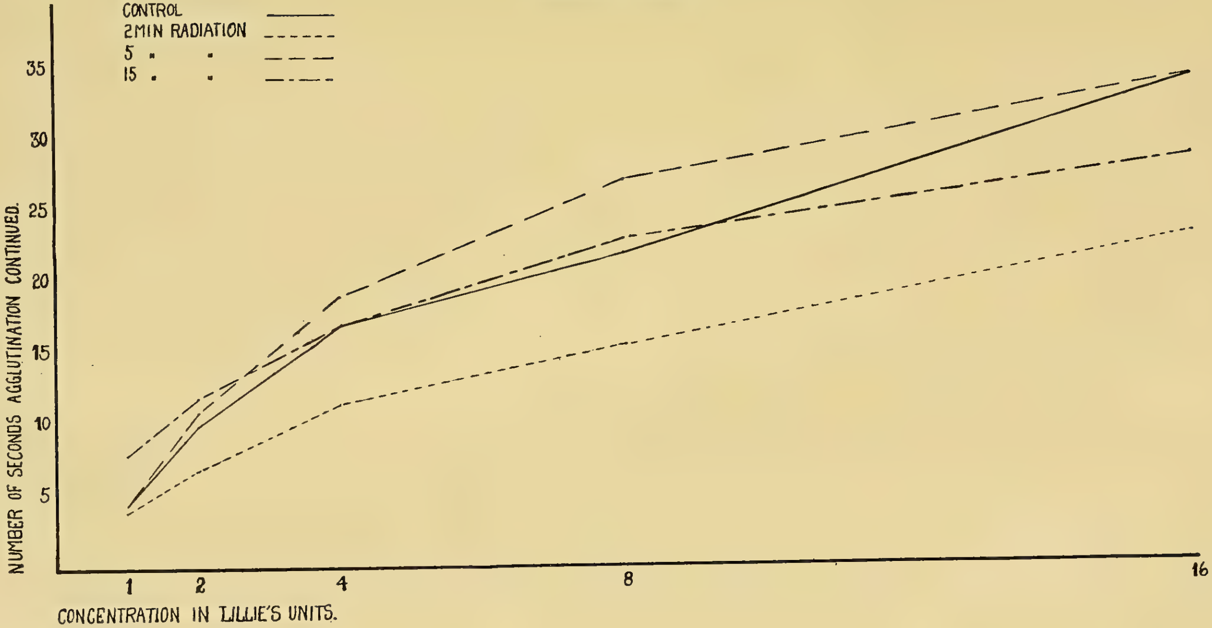

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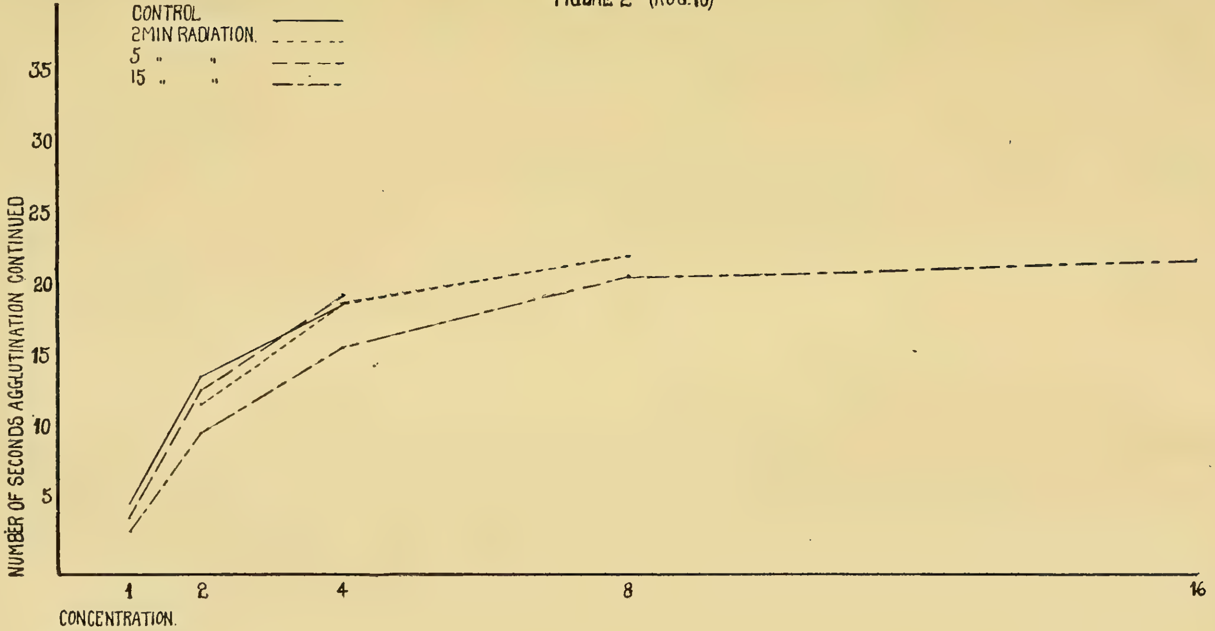
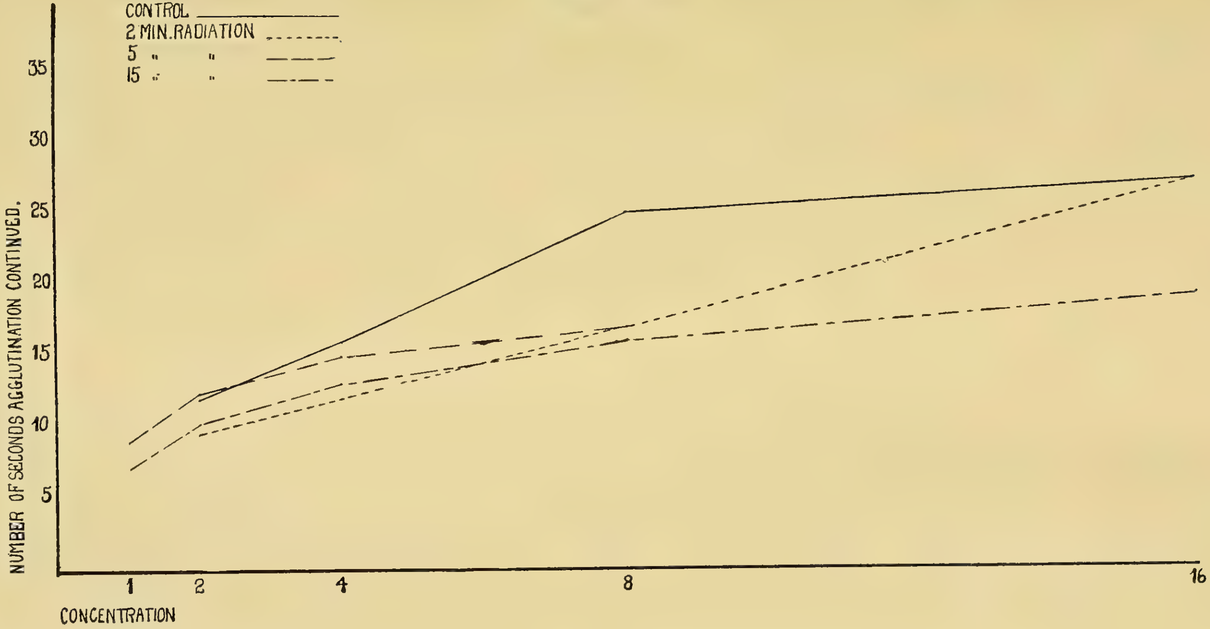
FIGURE 2 - (AUG. 10)



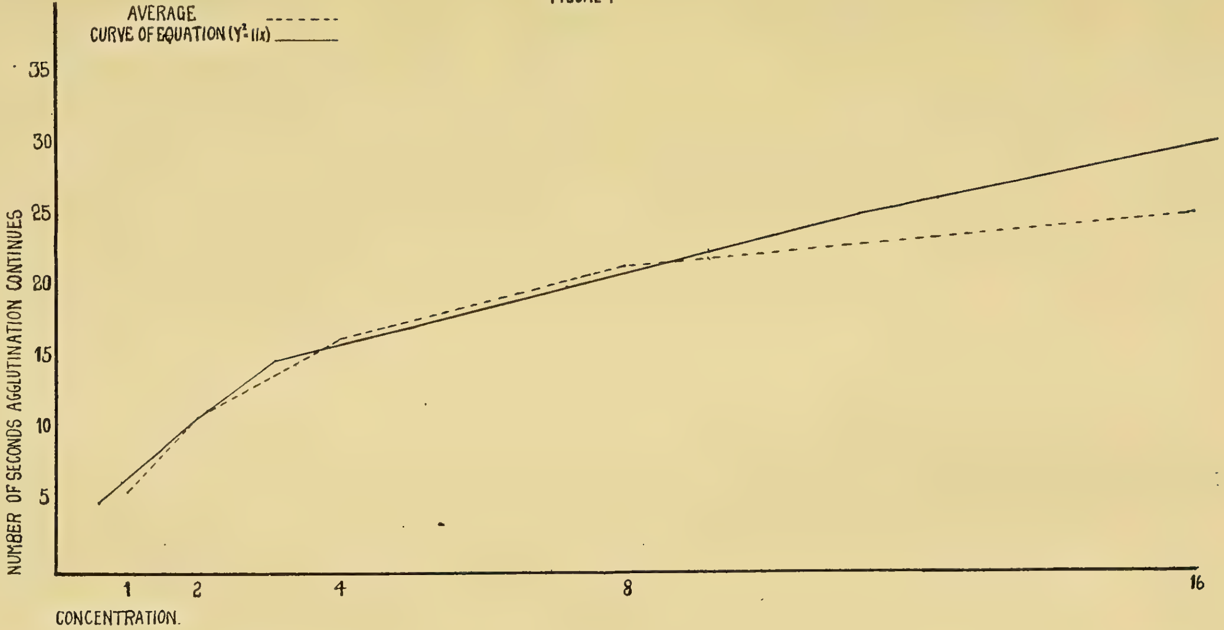
RICHARDS AND WOODWARD

FIGURE 3 – (AUG. 11)



RICHARDS AND WOODWARD

FIGURE 4 –



RICHARDS AND WOODWARD

CAN A SINGLE SPERMATOZOÖN INITIATE DEVELOPMENT IN ARBACIA?[1]

OTTO GLASER.

During the summer of 1913 while making the camera lucida tracings on which I have based my comparisons between the volumes of the unfertilized and fertilized ova of *Arbacia*,[2] it became necessary, in order to prevent rotation on the part of the eggs, and the consequent necessity of readjusting the focus, to employ very attenuated suspensions of sperm. The result of the highest dilutions used in these experiments, however, gave an unforeseen result since the appearance of the fertilization membranes was either very much delayed, or failed entirely to take place. This observation suggested the idea of a mass effect of the spermatozoa, and the possibility that this might play a rôle in normal fertilization.

At that time I had already made observations which had convinced me that the fertilization membrane in this egg is not formed de novo, but is preformed in the unfertilized egg, and simply rendered visible by changes occurring at the time of impregnation.[3]

The mechanism through which the fertilization membrane becomes visible will be dealt with in detail at another time; for the present it is sufficient to say that the absorption of water plays an important rôle. It occurred to me therefore that the prevention of this absorption and perhaps the prevention of fertilization itself might be possible even with the employment of more concentrated suspensions of sperm, if the eggs were first treated with Ca. As a matter of fact, it was either difficult or impossible to fertilize eggs so treated. The spermatozoa were active enough, but failed to enter, and fertilization membranes did not appear The following protocol is typical: In a small watch crystal, 4 volumes of fairly dense egg-suspension in sea-water+2 volumes n $CaCl_2$. After two minutes washed in sea-water.

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[1] From the Marine Biological Laboratory at Woods Hole, and the Zoölogical Laboratory of the University of Michigan.

[2] "The Change in Volume of *Arbacia* and *Asterias* Eggs at Fertilization," BIOLOGICAL BULLETIN, Vol. XXVI, pp. 84–91.

[3] "On Inducing Development in the Sea-Urchin (*Arbacia punctulata*), together with Considerations on the Initiatory Effect of Fertilization," *Science*, Vol. XXXVIII., pp. 446–450.

12.26 insemination moderate.
12.28 0 fertilization membrane.
12.30 1 " "
12.34 1 " "

Control normal. All eggs with fertilization membranes in 3–5 minutes after insemination. 100 per cent. cleavage. Hundreds of eggs examined in both control and experiment. In Ca-eggs very few divisions.

In connection with these experiments I noticed that insemination with great excesses of sperm frequently led to results at variance with the above, for fertilization membranes appeared about the majority of the eggs despite the use of Ca, and these eggs developed. This experience strengthened my belief, not only in the validity of the Ca-experiments, but also in the correctness of the original idea, namely that the number of spermatozoa that come into contact with the egg may make a difference.

Encouraged by this result, I diluted a sperm-suspension until only the faintest trace of opalescence remained. Several drops of this attenuated fluid were then drawn up into a medicine dropper of medium size and expelled quantitatively. If the dropper, which of course remained infected with sperm, was then used to agitate eggs in a small quantity of sea-water by carefully drawing the water in and expelling it several times, it was found that very soon a few spermatozoa had attached themselves to every egg. In an optical diameter, 4 to 5 sperm could easily be distinguished, but I awaited further changes in vain, despite the fact that the spermatozoa seemed to have reached the eggs, exhibited the usual amount of activity, and were potent in 100 per cent. of the cases when applied in larger quantities to eggs of the same lot. The following experiment is illustrative:

12.17 insemination with infected pipette.
12.18 0 fertilization membranes.
12.19 0 " "
12.20 1 " "
12.21 2 " "
12.22 3 " "
12.30 3 " "

Control normal; all eggs with fertilization membranes in 3 to 5 minutes. Experimental eggs examined at irregular intervals throughout the day, but no increase in the number of membranes.

Whether the appearance of a fertilization membrane, and impregnation itself will fail to take place in other eggs under similar conditions cannot be predicted, and is perhaps even improbable. With the eggs of *Arbacia punctulata* however I repeated these tests so often that I cannot doubt the correctness of my observations, and I therefore fail to understand Kite's[1] claim that he succeeded in calling forth a fertilization membrane in this egg by means of a single spermatozoön. I imagine that his method involved factors whose importance was unsuspected, since he says: "The real difficulty with this type of experiment is not the size of the spermatozoön, but the fact that when four or five are injected into the egg-jelly, they usually swim out and away from the egg. This necessitates the making of many injections in order to get a single spermatozoön to attach itself to the vitelline membrane and start the reaction." The "making of many injections" very likely involves touching the vitelline membrane an equal number of times, which recalls an experiment mentioned in my earlier paper[2] in which fertilization membranes were induced by surrounding the eggs with large numbers of minute infusoria. Observation indicated a continuous bombardment of the ova.

A quantitative relation between the rate of appearance of the membrane and the agencies, spermatozoa, normally calling it forth is really no more surprising than the efficacy of Ca as an inhibitor. Since now sea-water of sufficient hypotonicity will of itself call forth membranes[3] one may expect the exact reverse of the Ca-experiments if one immerses the eggs briefly in hypotonic solutions. Such ova, if not submerged too long so that the appearance of the membrane would have to be attributed to the hypotonic treatment itself, should be capable of fertilization by means of the sperm-infected medicine dropper. Actually under these circumstances fertilization with only 4 to 5 spermatozoa visible in the optical equator is possible in a considerable number of eggs.

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[1] G. L. Kite, "The Nature of the Fertilization Membrane of the Egg of the Sea Urchin (*Arbacia punctulata*)," *Science*, Vol. XXXVI., pp. 562–564.

[2] *Science, loc. cit.*

[3] In my preliminary communication (*Science, loc. cit.*) I considered the method of "inducing" a fertilization membrane in *Arbacia* by means of hypotonic sea-water new. Schücking however described this procedure in the year 1903. (*Arch. f. d. ges. Physiol.*, Vol. 97, p. 85.) The same method was used on *Arbacia* eggs by McClendon in 1910. (*American Journ. Physiol.*, p. 246.)

PROTOCOL.

In a watch crystal 3 volumes of sea-water+3 volumes of distilled. Added 1 volume of an egg-suspension in normal sea-water. At the instant when the first indications of membrane "initiation" were noticeable added 3 volumes of "double sea-water," *i. e.*, sea-water whose volume had been reduced one-half by boiling. By means of a sperm-infected pipette every egg was provided with 4 to 5 spermatozoa. In a series of microscopic fields the number of undivided eggs was later compared with the number that had divided. The results were:

Experiment I.		Experiment II.	
Undivided.	Divided.	Undivided	Divided.
7	2	4	1
4	3	3	2
4	4	12	2
7	2	18	0
1	2	6	5
6	0	6	0
2	4	7	4
5	1	8	0
5	4	8	5
5	2	14	4
3	2	14	3
5	3	12	3
5	3	13	1
6	5	8	2
2	6	6	3
4	3	7	1
		13	2
		6	1
		6	3
		10	1
		8	2
Total...71	46	189	45
Per cent.61	39	81	19

Controls: Normal eggs+usual amount of sperm = 100% Fertilization. Eggs treated as above+usual amount of sperm = 100% Fertilization. This is in sharp contrast with the earlier experiments in which the operations were carried out at the same dilutions but without the brief fore-treatment with hypotonic sea-water. Results which harmonize with these but prove less satisfactory on account of injuries to the eggs can be gotten by the use of heat. In this case one might think of a parthenogenetic effect, but in *Arbacia* at least, it is not easy to confuse the usual parthenogenetic cleavage with normal two or four-cell stages.

It is very easy to misunderstand these experiments and to draw wrong conclusions. There is no more doubt in *Arbacia punctulata* than in any other form that a single spermatozoön is sufficient to carry out the biparental effect. Furthermore the experiments with dilute sperm do not in anyway enable us to prejudge what would happen in another egg under similar conditions nor do they warrant the inference that the initiation of development by a single sperm is impossible in *Arbacia* ova deprived of their superficial coverings. I feel very sure of this however: In *Arbacia* the appearance of the fertilization membrane after insemination is a sign that the egg investments have allowed the sperm to pass through. This passage has been possible because the coverings have changed. The change depends on a synchronous softening and absorption of water, the latter having consequences as the result of which the membrane becomes visible. Inasmuch as the becoming visible of the membrane is a reliable index of fertilization, and one of the consequences of fertilization is the division of the ovum, we may say that the initiation of development by a single spermatozoön in this case is impossible because a single sperm cannot effect those changes in the egg-coverings which will permit it to reach the protoplasmic surface film that lies beneath. The situation is exactly as though the entrance to a room were blocked by a barrier which a single man could not break down, although a group of ten might. Once broken down, any one of the men could cross the threshold, but for the opportunity of doing this, the services of the others would be needed. With this analogy in mind, the statement that a single spermatozoön cannot except possibly under special conditions, fertilize the normally invested egg of *Arbacia punctulata*, would appear to agree with the facts.

Zoölogical Laboratory,
University of Michigan,
November 12, 1914.

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL. XII.

On an Abnormality of the Oviduct and Its Effect upon Reproduction.[1]

MAYNIE R. CURTIS.

In a recent paper Pearl and Curtis (1914) have shown that when the passage of an egg through the oviduct is prevented by surgical interference with the duct the sex organs pass through their normal reproductive cycles. The oviduct functions to the level where the passage is interrupted and the egg is then returned into the body cavity. The eggs thus set free may be absorbed without causing any serious disturbance in metabolism. In a paper still in press (Curtis and Pearl) it has also been shown that congenital or acquired obstructions to the oviduct may occur without artificial interference and that the results in such cases are the same as in the former cases.

The following case was recently brought to our attention by Mr. J. C. Hawkes, Poultryman at the Maine Agricultural College poultry plant.

A year and a half old Rhode Island Red bird was killed for meat. She was well grown, in good flesh and in every respect was perfectly normal in appearance. When an incision was made to remove the viscera a full sized membrane shelled egg slipped into the opening. Mr. Hawkes then kindly turned the bird over to us for examination.

The eggs and egg membranes shown in Fig. 1 were all removed from the body cavity of this bird. These represented every possible stage of absorption of the egg from a normal membrane shelled fresh egg to the collapsed empty membranes shown in the fourth line of the figure. Some of the eggs and some of the empty membranes were free in the body cavity. Some were partly or entirely enclosed by peritoneum. In several instances two eggs or an egg and a bunch of membranes were walled off together. The last line of the figure shows collections of empty membranes enclosed in peritoneum. These peritoneal covered masses were attached by suspending strings or folds of peritoneum. The large mass at the right end of this line contains a very large number of these empty membranes. A larger view of it is shown in Fig. 2. The second line from the bottom of Fig. 1 shows collapsed empty egg membranes of which some are single and some two or three tightly packed together. The three top lines of the figure show eggs in various stages of resorption. One was a normal fresh egg in a single egg membrane. Ten had evidently been normal eggs but at the time of autopsy they contained a homogeneous mixture of yolk and albumen which had lost the gelatinous character of fresh egg albumen. Each of these eggs was enclosed in a single egg membrane. The other four eggs were double eggs. These eggs were much like the double eggs (*ovum in ovo*) described by Parker (1906), Patterson (1911) and by many other writers. (The appended bibliography is supplementary to the one given by Parker 1906.)

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[1] Papers from the Biological Laboratory of the Maine Agricultural Experiment Station No. 76.

The eggs of this sort described in the literature had all been laid. Most of them have had shell on one or both of the concentric components. The double eggs found in the body cavity of this Rhode Island Red hen had no shell on either the enclosed or enclosing egg. The nature of the contents of the double eggs differed in each of the four cases. In one both enclosed and enclosing egg contained yolk. The yolk and albumen of the enclosing egg were somewhat mixed, although they did not yet constitute a homogeneous fluid. In fact the currents or streams of yolk could be seen in the clear albumen through the semitransparent egg membrane. The yolk and albumen of the enclosed egg were still more distinct although the yolk membrane had already ruptured. The enclosed egg was about the size of the normal egg and the enclosing egg (the third egg in the top line) was the largest egg found in the body cavity. A second double egg was composed of a normal sized enclosed egg which had apparently contained the normal egg parts. The contents had, however, been reduced to a homogeneous brownish-yellow liquid much thinner than fresh egg albumen. The enclosing egg was only slightly larger than the egg it enclosed and it seemed probable that a second egg membrane had been received directly around the first on its passage back up the duct. A third of the double eggs had two closely applied egg membranes as in the preceding case but the enclosed egg was itself a double egg. The inner egg in this series was a small "witch" or "cock" egg containing a little yolk not enclosed in yolk membrane and a small amount of normal fresh albumen. The outer egg contained only normal fresh albumen. The other double egg was even more remarkable in character as it consisted of a concentric series of four enclosed eggs. The inner one, like the inner egg just described, contained a little free yolk enclosed in normal albumen. Each of the successive enclosing eggs contained only normal albumen. This whole egg was not larger than a normal hen's egg.

These peculiar double formations indicate that an egg did not always pass up the duct in time to get out of the way of a succeeding egg. In case an egg met another yolk it might become enclosed in a double egg or it might change the direction of the incoming yolk If the yolk was ruptured and a part remained in the duct it might furnish the nucleus for a "cock" egg which might then become enclosed in a succeeding egg. Apparently the direction of peristaltic movements became at times much disturbed, as the last double egg described must have passed up and down the duct several times before it was finally extruded into the body cavity.

The visceral organs of the bird were in normal condition. There was a little slightly oily yellowish serous fluid bathing the viscera. The peritoneum was very slightly thickened but otherwise normal. The ovary was normal with a normal series of enlarging yolks and resorbing follicles. It was apparent that the bird was in the midst of a normal reproductive period and was backing membrane shelled eggs into the body cavity and resorbing them with great rapidity.

The oviduct (Fig. 3) was perfectly normal from the funnel mouth to the posterior end of the isthmus. Here the tube abruptly ended blindly at *D*. There was no shell gland or vagina.

The oviduct ligaments were continuous to the posterior end of the body cavity. That is the tube ended in the fold of enclosing peritoneum while the fold continued to the posterior end of the body cavity. The heavy bands of smooth muscle in the ventral ligament (see *E*, Fig. 3) continued to the end of the body cavity —several centimeters beyond the end of the tube. The tube rounded off smoothly at the posterior end and the ligament behind did not present the slightest indication that it had ever contained any oviduct tissue. It seems probable that the duct had never extended any farther than at present. From the embryonic history of the oviduct it is evident that if the actively growing point of a duct should cease at an unusually long distance anterior to the cloaca a blind oviduct of this form might result.

The development of the oviduct according to the account given by Lillie (1908) begins on the fourth day of incubation as a groove-like invagination of a strip of thickened peritoneum on the surface of the Wolffian body or embryonic kidney. The lips of this groove fuse on the fifth day so as to form a short tube open anteriorly to the body cavity and ending blindly posteriorly. The open end of this tube becomes the *ostium tubæ abdominale* or funnel mouth of the oviduct. The posterior end grows backward between the strip of thickened peritoneum and the Wolffian body. It normally reaches the cloaca on the seventh day. The growing point is always a short solid wedge of cells. The duct receives its lumen a short distance anterior to this. On the twelfth day of incubation the primordium of the shell gland is distinctly visible as an expansion of the lower end of this tube.

The most probable explanation of the abnormality of the oviduct found in the case described is that in early embryonic development (probably on the sixth or seventh day of incubation) the backward growth of the primordial oviduct stopped permanently while the differentiation of the part already formed continued in the normal manner.

As in other cases where the passage of the egg is prevented the sex organs passed through their normal reproductive cycles; the oviduct functioned as far as the point where the passage was interrupted; the eggs were then returned to the body cavity and resorbed. The number of eggs and empty egg membranes found in this fowl which was apparently in a perfectly normal physical condition show that a bird possesses very great power of resorption of its own proteins from the peritoneal cavity. Such resorption does not necessarily cause metabolic disturbances.

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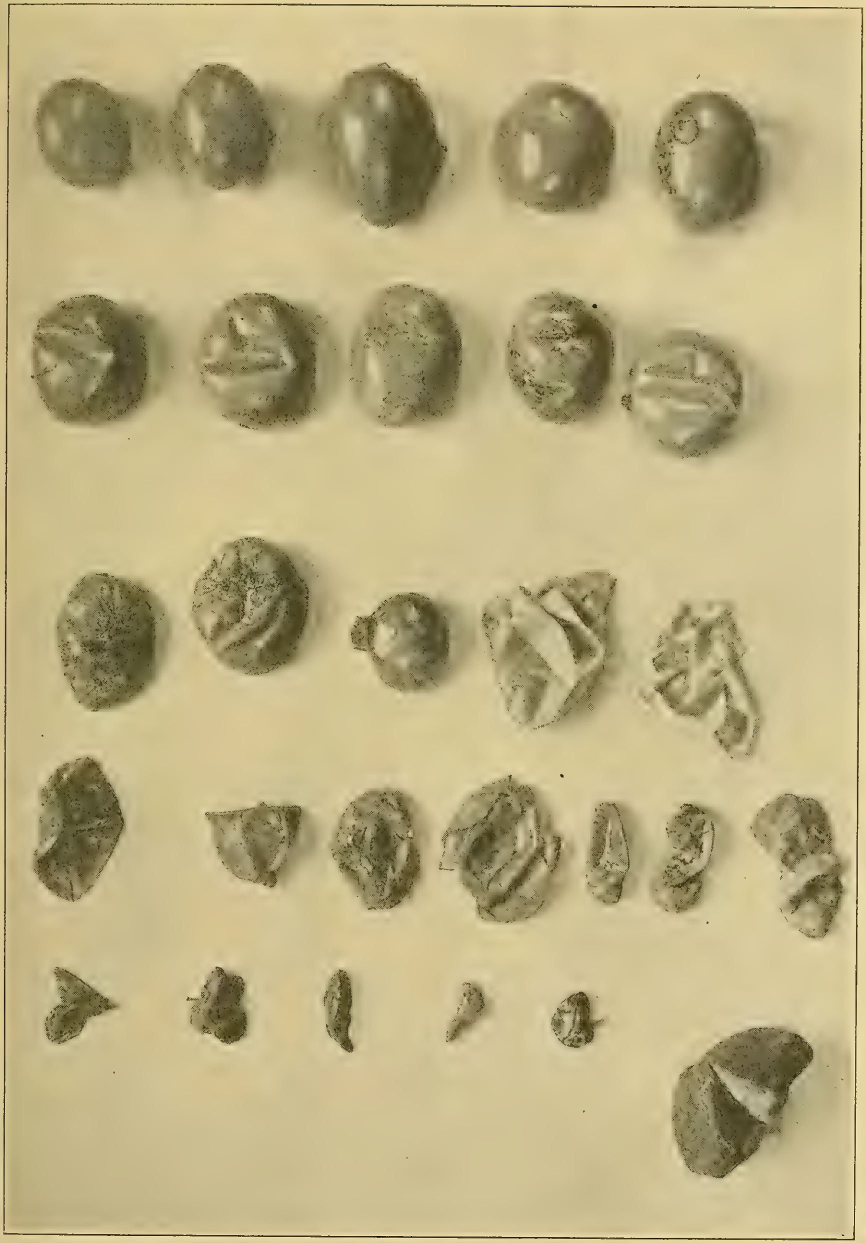
Explanation of Plate I.

Fig. 1. Eggs and egg membranes removed from the body cavity of a Rhode Island Red fowl.



MAYNIE R. CURTIS.

FIG. 1.

Explanation of Plate II.

Fig. 2. Natural size photograph of the large peritoneal covered mass of egg membranes shown at the lower right hand corner of Fig. 1. This is cut across and opened back to show its composition.

Fig. 3. Photograph (greatly reduced) showing the oviduct of the bird from which the eggs in Fig. 1 were taken. *A* =funnel; *B* =albumen secreting region; *X* =isthmus ring; *C* =isthmus; *D* =blind end of the oviduct; *E* =mass of smooth muscle in ventral ligament posterior to the end of the oviduct.

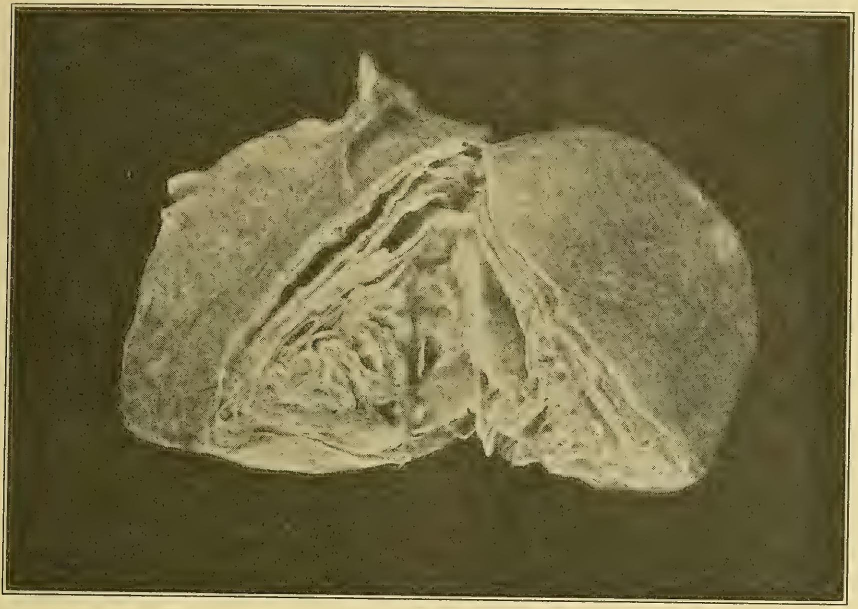
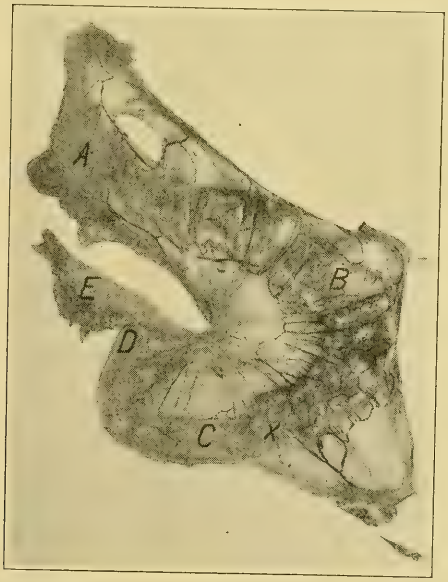


FIG. 2.



MAYNIE R. CURTIS. FIG. 3.

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BIOLOGICAL BULLETIN

LINKAGE OF CHROMOSOMES CORRELATED WITH REDUCTION IN NUMBERS AMONG THE SPECIES OF A GENUS, ALSO WITHIN A SPECIES OF THE LOCUSTIDÆ.

Smithsonian Institution MAY 5 1915 National Museum

CARRIE I. WOOLSEY.

In the following paper it is my purpose to give the result of my study of the chromosome numbers within three species of one genus of the Jamaican *Locustidæ.*

The material used was collected in Jamaica during the summer of 1912 by Professor W. R. B. Robertson, of Kansas University. The testes of all but one individual, No. 416, were removed while in the field and fixed in either Bouin's or Flemming's fluid. The slides were made in the fall of 1912. The clearest and best division figures as well as those in which the chromosomes were most crowded together and unsatisfactory were made from Flemming-fixed specimens. The sections were cut at about twelve micra and iron-hematoxylin proved most satisfactory for staining. All drawings have been made at the level of the base of the microscope with the aid of a camera lucida. A 2 mm. Spencer oil immersion lens and No. 18 Zeiss compensating ocular were used. All the figures were magnified to 3,900 diameters and have been reduced 1/5, giving a final magnification of 3,120 diameters.

The specimens studied were identified and classified by Mr. A. N. Caudell of the United States National Museum, Washington, D. C., as follows:

588 and 589, adult males of *Jamaicana flava*, N. Sp., Caudell.

430, a nymph and 560, an adult male of *Jamaicana unicolor*, Brunner.

416, 585, 586, 587, four adult males of *Jamaicana subguttata* Walker.

438, a nymph and 503, a small male of *Jamaicana subguttata*.

In determining the number of chromosomes in the various individuals, I have, in all cases, made drawings from not only different cysts, but from different follicles as well. In a few instances where the figures are particularly clear and distinct, I have taken more than one cell from the same cyst (Nos. 30, 31, 33) but where this was done I made additional drawings from other parts of the testes. There can be no doubt as to the character or appearance of these figures.

Observations.

Jamaicana flava n. sp. Caudell.

All chromosomes are of the rod type. One individual shows two pairs of peculiarly associated chromosomes in the spermatogonial division figures.

In my comparison of species, the description of *J. flava* should logically come first since the chromosomes here are all of the rod shaped, simple type, and are not sufficiently associated to form multiples. They also vary the least in their behavior from what we might consider the original or primitive condition of the species of this genus. To distinguish individuals of the same species, I shall refer to each by number. Nos. 589 and 588 belong to *J. flava*.

In individual 589, I found the number of chromosomes to be thirty-five. They are all rod-shaped, varying in size from the large unpaired accessory chromosome which I have numbered 18, through a graded series of pairs indicated by number, according to their size from 17 to 1.

In a polar view of a spermatogonial metaphase figure, the seventeen or eighteen largest chromosomes are found, as a rule, on the periphery in no fixed position or order, with the smallest pairs clustered about in the center. This is quite well shown in Figs. 3 and 4 while Figs. 1 and 2 show some of the larger chromosomes in the center of the figure where they have probably displaced the smaller ones through some accident in previous mitoses. There seems to be nothing unusual or irregular in the number or relation of chromosomes here either in spermatogonial or spermatocyte divisions.

In the first spermatocyte stages the chromosome complex consists of seventeen undivided autosomes plus the accessory or sex-chromosome (Figs. 10 and 11). As division proceeds, the unpaired sex-chromosome goes entire to one pole accompanied or followed by 17 autosomes. The mates of these seventeen autosomes pass to the opposite pole, thus making the number at the two poles eighteen and seventeen respectively. Before this division takes place, the paired autosomes show the same gradation in size as was noted in spermatogonial figures (Fig. 11). There are several extremely large chromosomes in this individual but the change in the series is so gradual that it is difficult to say where the dividing line falls. However, I am reasonably sure I can pick out at least three pairs in the spermatogonial or three undivided chromosomes in the first spermatocyte cells, that are larger than the others.

In 588, the second individual of *J. flava* studied, a slight but distinct difference can be noted in the spermatogonial figures. The number of chromosomes is the same, thirty-five, and all are of the rod type varying in size as was noted in No. 589. However, in pairing off the chromosomes, I find there are in each cell, two pairs of closely related ones. Each member of the pair No. 16 is always found in close association with a member of the pair No. 14 (*A*, Figs. 5, 6, 7, 8). The members of the other pairs are distributed throughout the figure very much the same as in corresponding figures of No. 589. The largest chromosomes always appear on the periphery and the smallest ones in the center. These two sets of parallel rods, as may be noted, in the figures, have no regular position in respect to each other or to other chromosomes in the cell. Sometimes they are near each other and as often they are to be found on opposite sides of the figure. However, they are never far from the accessory chromosome.

Jamaicana subguttata Walker.

Five individuals out of six belonging to *J. subguttata* show only the rod type of chromosomes. In the sixth individual, a distinct variation is found in the appearance of a V-chromosome.

Of the ten individuals I studied, numbers 416, 585, 586, 587, 438 and 503 belong to *Jamaicana subguttata*. The sections of

No. 416 broke up badly so that it was difficult to find a perfect division figure. However, a sufficient number were found to determine the number and character of the chromosomes (Figs. 13–14).

I found no distinctive feature in the number, arrangement, or behavior of the chromosomes in the first five individuals of this species (Figs. 13 to 29). There are thirty-five chromosomes in each and their appearance is very similar to that noted in individuals, No. 588 and 589. The accessory chromosome is very prominent and in the majority of spermatogonial metaphase figures it is found with seventeen of the largest autosomes on the periphery, the remaining seventeen small ones being in the center of the figure (Figs. 13, 15, 25–29).

A most interesting feature was found however in the individual No. 503. Instead of the thirty-five autosomes of the simple rod type that were common to the other members of the genus as well as species, I found here thirty-three rods and a large V-multiple whose arms are of unequal length. When I attempted to arrange the autosomes in pairs, I found two of the large ones, numbers 16 and 14, without mates among the rod type but corresponding in size to the arms of the V (Figs. 30 to 33). In one cyst I found several perfect cells in spermatogonial metaphase showing this size relation between the rods and the arms of the V (Figs. 30–31–33).

After satisfying myself that this multiple chromosome appears in all spermatogonial metaphase figures, I examined cells in other stages of growth. In the various phases of the first and second spermatocyte cells I found the V still present. In the first spermatocyte figures the rod mates were often still attached to the arms of the V but the break could always be distinguished more or less distinctly (Figs. 37 through 50). In first spermatocyte metaphase (Fig. 42), I found the V still attached to or united with its mates as were several of the rod chromosomes also. In this stage I found fifteen rods without the two in the V and the accessory, making in all eighteen chromosomes in the first spermatocyte.

In the second spermatocyte division the accessory chromosome divides longitudinally so that each daughter cell receives half.

The same kind of division evidently takes place in the V-multiple here. In Fig. 52, we can see the one V going over to one pole a little after the other chromosomes have passed. What appears to be the other half of the V has already reached the opposite pole. I did not find another cell showing this conclusively but the division of the other parts of the chromatin matter would seem to indicate this interpretation. The V at the pole is again the size of the multiple in the spermatogonial stage.

Jamaicana unicolor Brunner.

The two individuals of *J. unicolor* differ. One is of the simple rod type, while the other shows the two V-chromosomes.

The spermatogonial metaphase figures of individual 430.1 show thirty-five chromosomes of the rod type (Figs. 54–57). There is nothing in their appearance or behavior to distinguish them from individual 589 of *J. flava* or from individuals 416, 585, 586, 587 and 438 of *J. subguttata* and the same holds true in later stages of growth.

In No. 560.1, the second individual of this species, I found two multiple chromosomes (Figs. 58–67). In addition to the thirty rod autosomes, the accessory and two large V's are to be seen on the periphery of the spermatogonial metaphase figures. The chromatin material in this series of slides is so badly massed that perfect cells are difficult to find. For this reason I can not be certain of the size relations among all the chromosomes but I have gained the essential facts for my purpose. The largest chromosomes are quite clear and distinct so that I can number the mates of the five largest pairs besides the accessory. Figs. 58–60 show the number and arrangement of the chromosomes. Many spermatogonial cells can be found in which the two V's and the accessory are readily distinguishable although the other chromosomes may be too badly massed for further study (Figs. 61–64).

In the first spermatocyte, the multiples are found attached to each other end to end, thus forming an elongated ring. A slight constriction appears where the ends of the V's meet (Figs. 65–66).

The cells in the earlier growth stages were small and the chromatin so massed together that I gained nothing from them.

DISCUSSION.

McClung and others who have worked on the chromosomes of the *Orthoptera* believe there is a fixed or definite number for each group of related insects, and presumably for all kinds of life. Other writers disagree. Miss Browne ('13) has summarized the work done and results obtained by many investigators on varied kinds of life so thoroughly that I shall not go into this in detail. I shall, however, review the methods as set forth by her whereby the changes in the chromosome numbers have been accounted for by the various authors.

One method is by the fusion or separation of particular chromosomes. Miss Browne in her work on *Notonecta* ('13) and Wilson ('11) on *Nezara* use this explanation. A change in number by a process of fusion was used by McClung ('05) and Robertson ('15) in the appearance of a multiple chromosome, the former in *Hesperotettix* and *Mermiria*, the latter in *Chorthippus* (*Stenobothrus*) *curtipennis*. A change by a process of splitting has been advocated by several observers, Payne ('09) and Wilson ('11) among them. These account for slight or gradual changes but wide variations are accounted for by Wilson by a new segregation of the nuclear material causing a change in number and size relations of the chromosomes, but not in their essential quality. Another method whereby a change might take place is by an abnormality occurring in mitosis. Wilson ('09*a*) has described an unequal distribution of the chromosomes to the daughter cells in *Metapodius*. An arrest of cell division after a division of the chromosomes has taken place was found by Boveri ('05) in sea-urchin eggs.

My material resembles McClung's ('05) in that the change in number is accounted for by the fusion of chromosomes, these giving rise to a multiple (V). However, the composition and behavior of this multiple differs from the one described by him in so far as its relation to the sex chromosome is concerned.

These multiple or V-chromosomes resemble very much more those being described by Robertson ('15) in *Chorthippus* (*Steno*) *bothrus*). Robertson has found that *Chorthippus* (*Stenobothrus-curtipennis* has seventeen chromosomes and of these, six are V's (three pairs of V's). Counting each limb of the V as a chromosome, he believes there results twenty-three, the number normal for *Truxalinæ* a subfamily of the *Acrididæ*. It was at his suggestion that I undertook to determine if *Jamaicana* which I found to have these V-shaped chromosomes showed the same phenomena.

In my material the multiple is likewise apparently formed by the union of two of the autosomes. As has been done by others, I numbered and paired the chromosomes according to size, calling the unpaired accessory which is the largest chromosome in the complex, No. 18, and grading the others down from that. I at once found two autosomes of unequal length, No. 16 and 14, without mates as well as the very noticeable V. Since the arms of the V are unequal, and correspond in size as well as length to the unmated rods, I believe No. 16 is the mate of the long arm of the V, while No. 14 mates with the short arm of the multiple (Figs. 30–36). The sex chromosome is very prominent and is often seen in close proximity to the multiple throughout the transitional stages, but it never unites with the components of the multiple as McClung found in *Hesperotettix* and *Mermiria*.

In the spermatogonial metaphase cells the V first appears. It is always on the periphery of the ring with the apex pointing inward, while its rod mates may be anywhere on the periphery, sometimes near it, sometimes opposite it.

From the spermatogonia through the spermatocytes this multiple can be traced. It divides longitudinally in spermatogonia. In the first spermatocyte the tetrads divide transversely separating the V from its rod mates. Half of the second spermatocyte cells receive the V and half receive the rod mates of the V (Figs. 37–47). In the second spermatocyte we have every reason to believe that the V divides longitudinally, each of the two spermatids resulting thus receiving a V while from the other second spermatocyte two spermatids result which will contain the rod mates of the V.

This is what takes place in the one V-type of *J. subguttata*. The associated rods in No. 588 *J. flava* (Figs. 5–8) are easily distinguishable in the polar view of the spermatogonial metaphase and their size 16 and 14 in the series corresponds to that of the arms of the V in No. 503. Their behavior can not be traced as readily as the V, however, since their union is evidently not permanent and the rods separate in the later growth stages. The fact that they are associated in the early stage forming two sets of related rods on the periphery strengthens my belief in the manner of the V-formation in the other forms. In the place of these two sets of rods, or of the one V and one set of rods in No. 503, I found two V's in No. 560 a representative of *J. unicolor*. Here we find the same thing taking place as in the one V type. In the place of a long V formed by the short V and the rod mates in the spermatocyte figures, we here find a ring formed by an end-to-end union of the arms of the V's (Figs. 65–67).

It will be seen that throughout the three species of this genus of Jamaican *Locustidæ* the number of chromosomes, 35 remains constant, although their behavior varies not only within the genus but even within the species. In seven of the ten individuals studied representing three species, there are 35 chromosomes of the simple rod type. As a whole, the division figures are clear and distinct and the growth stages can be followed with moderate certainty. In the first spermatocyte, the accessory passes undivided to the one pole making the number in the two daughter cells 18 and 17 respectively. When these divide in the second spermatocyte, division takes place longitudinally and the spermatids number 18, 18, 17 and 17 respectively, the last two being without the accessory chromosome. These then will produce males when fused with eggs, and the oosperm will contain thirty-four autosomes plus the one sex chromosome brought in by the egg. Those spermatids with eighteen, will produce females, since here there will be two sex chromosomes, one from the sperm and one from the egg in addition to the thirty-four autosomes, making the number 36 in the females.

I find, however, an exception from this general rule of 35 separate rod type chromosomes in each species studied. *J. flava* shows in one individual the presence of two pairs of associated rods in all spermatogonial figures. The rods that make up each pair or set are unequal and hence can not be mates. In numbering the members of the complex these rank as 16 and 14 in the graded series, 18 being the unpaired accessory and the largest in the group (Figs. 5–8). I find by numbering the members of the pairs as separate individuals, we have thirty-five chromosomes here as in the other seven forms. In the later stages of growth, nothing appears to distinguish this form from its fellow No. 589 so we must conclude the rods of the related pairs are not held permanently together but are lost after the spermatogonial division among the other rods of the complex.

In *J. subguttata* a multiple chromosome appears resembling a V which I believe has been formed by the union of two of the autosomes. These, by continued association, have finally united end to end and we find them forming a V. The members or arms of the V are unequal and, as was noted in connection with the associated rods, can not be mates, since mates are identical. Hence the mates to the components of the multiple must be rods. I numbered and paired the chromosomes in the spermatogonia according to size and found two rods 16 and 14 without rod mates. These correspond with the arms of the V in size and appearance, and as many cells show the same relation, it seems to me they may be so taken. Figs. 30–36.

The inequality of the parts of the multiple is especially well shown in the first spermatocyte figures. The long arm of the V is linked up with a long rod of approximately the same size, while a somewhat shorter or smaller rod is united with the smaller arm of the V. A slight constriction is to be seen where the rods join the arms of the multiple.

In determining the count for this species, those individuals having the simple rod type chromosomes, have of course thirty-five in their complex. In No. 503, the odd one in this group, there are thirty-three chromosomes of the rod type plus the multiple which I believe is composed of two of these autosome rods united. This then makes the number for the entire species thirty-five, the same as was found for *J. flava*.

The exception in *J. unicolor* contains two multiples or V's similar to the one found in *J. subguttata*. They are apparently formed in the same way and probably of the same two chromosomes, Nos. 16 and 14, as the difference in the lengths of the arms of the V's corresponds with that in No. 503. In the first maturation division, the V's which compose the elongated ring, break apart at the constriction and each cell receives one V. This splits longitudinally in the next division so that each spermatid carries one V multiple.

J. unicolor contains thirty-one rods and the two multiples. If we accept what has apparently taken place in the previous cases, we can now say the V multiples are composed of four rods which will make the number for the individual thirty-five.

It seems evident then, that the ten individuals, representatives of three distinct species, contain a uniform number of chromosomes regardless of the fact that their behavior differs by the appearance of multiples in two specimens and a transitional form containing associated rods, not yet forming a V, in one individual.

Tabulated Results.

Species.	Accession Number of Specimen Studied.	Number of Free Rod-type Chromosomes.	Number of Groups of Rods Associated by 2's.	Number of V's.	Total Number of Rod Elements (Chromosomes).
J. flava	588	31	2	none	35
	589	35	none	none	35
J. subguttata.	416	35	none	none	35
	438	35	none	none	35
	503	33	none	1	35
	585	35	none	none	35
	586	35	none	none	35
	587	35	none	none	35
J. unicolor.	430	35	none	none	35
	560	31	none	2	35

Although I made my drawings by the aid of a camera lucida I determined the series of size relations from the slide as well as from the drawing, comparing and judging as accurately as I could the graded pairs. As a result of this study, I believe it is the same 14–16 pair that we find associated in the individuals described, Nos. 588, 503, and 560. Just what significance this may have upon the life processes of these grasshoppers I can not say. As to outer body characters, there is nothing to indicate a variation from those of the simple rod type.

There is still another interesting association in this material that I feel should be noted here. In the cases where a multiple is present, it is more or less closely attended by the extremely large accessory chromosome. In the No. 503 material, this really occurs more often than my drawings would indicate. Whenever the multiple is seen, whether in an entire cell or only in a section, it is quite rare that the sex chromosome does not accompany it. In the majority of cases, although my drawings do not bear me out in this, spermatogonial figures show these two elements on the same half of the periphery. The No. 560 material shows this particularly well. They are not connected as McClung found in *Mermiria* ('05), and Robertson in *Chorthippus* ('15) but seem to be influenced or attracted by each other so that they are generally found in close proximity.

It seems to me the individuality or genetic continuity of the chromosomes which Wilson ('09) speaks of, is pretty well established here in at least the multiple types. In cysts that show different stages of division, the V may often be distinguished still intact, so that it can be traced from spermatogonia through the second spermatocyte still in the V form. It is quite likely only one half the spermatids of the one-V type receive a V while each spermatid of the two-V type receive one.

I wish to express my thanks and indebtedness to Prof. W. R. B. Robertson for his helpful suggestions and encouragement during the progress of this work.

Zoölogical Laboratory,
Kansas University,
September, 1914.

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PLATE I.

Jamaicana flava n. sp. Caudell.

In polar views of spermatogonial metaphase figures, the number of chromosomes in both individuals is thirty-five. Chromosomes are paired and numbered according to size, 18 being the single accessory.

FIGS. 1–4. Polar view of spermatogonial metaphase of individual No. 589.

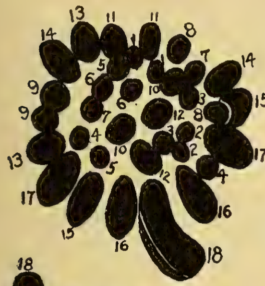
FIGS. 5–8. Polar view of spermatogonial metaphase of individual No. 588, showing, *A*, the oddly related pairs of chromosomes (14's and 16's) in each figure.

FIG. 9. Lateral view of spermatogonial anaphase. No. 588.

FIG. 10. Polar view, first spermatocyte of individual 589.

FIG. 11. Lateral view of metaphase of first spermatocyte chromosomes numbered according to size. Sex-chromosome going undivided to one pole. Individual 589.

FIG. 12. Prophase of second maturation division or interkinesis.



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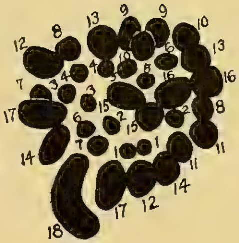

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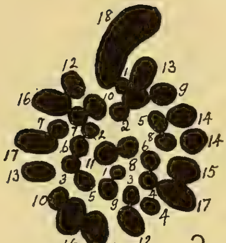

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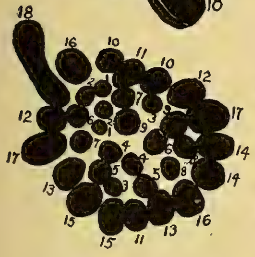

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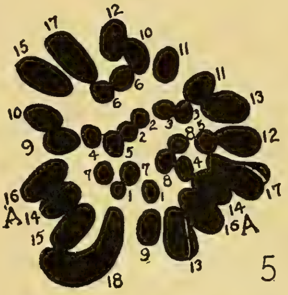

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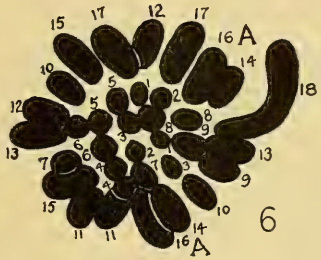

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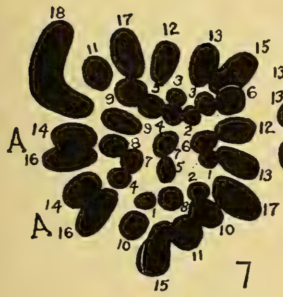

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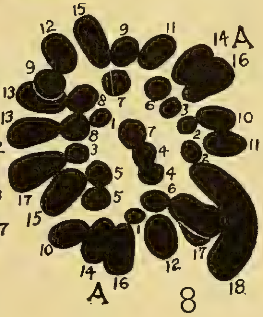

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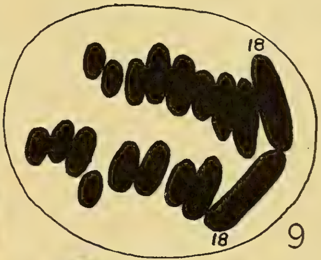

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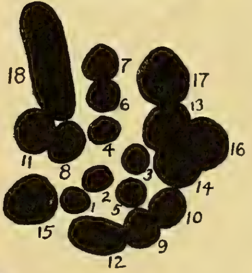

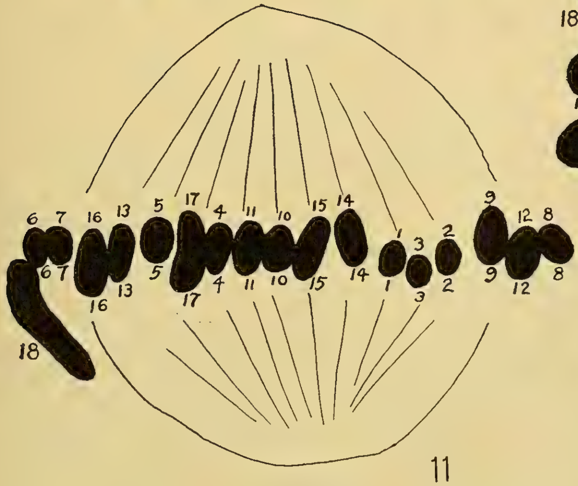

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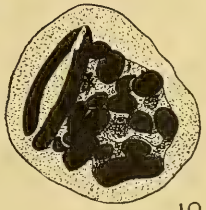

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C. I. WOOLSEY.

PLATE II.

Jamaicana subguttata Walker.

Spermatogonial figures from four individuals (Fig. 19 exception). The number in each polar view is thirty-five. Chromosomes are paired and numbered according to size, 18 being the accessory.

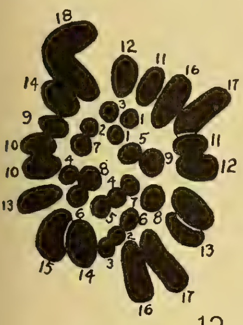
FIGS. 13–14. Spermatogonial figures from individual 416. Polar view, metaphase.

FIGS. 15–18. Polar view of spermatogonial metaphase figures from individual No. 585.

FIG. 19. Lateral view of first spermatocyte. The full number of chromosomes is not shown.

FIGS. 20–22. Polar view of spermatogonial metaphase figures from individual No. 586.

FIGS. 23–26. Polar view of spermatogonial metaphase figures from individual No. 587.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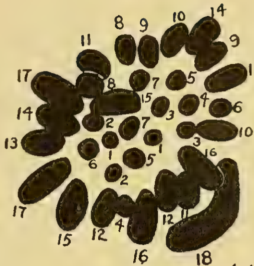
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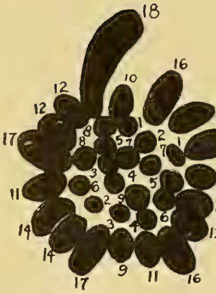
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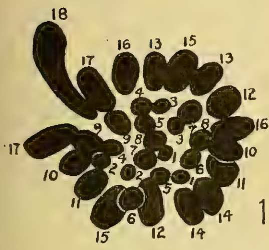
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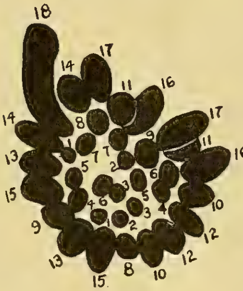
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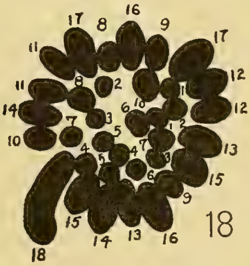
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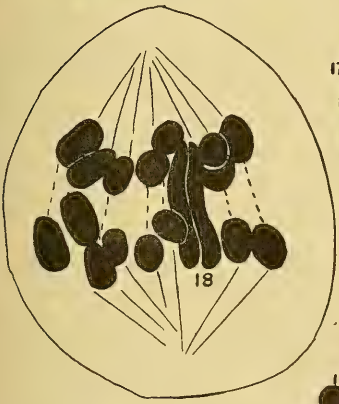

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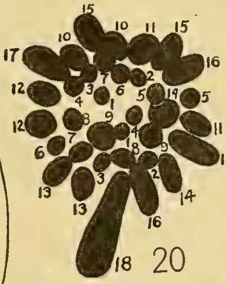
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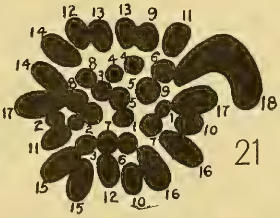
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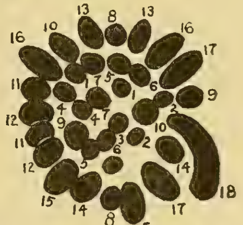
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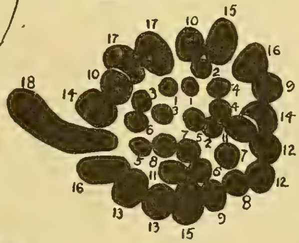
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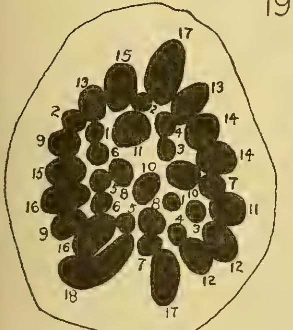
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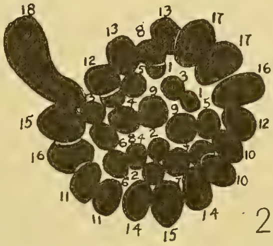
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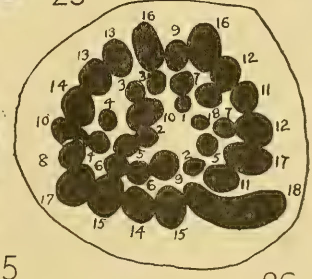
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Plate III.

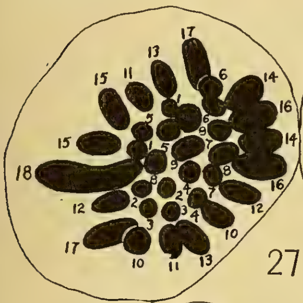
Jamaicana subguttata Walker.

Spermatogonial figures from two other individuals of this species. The number of chromosomes in 438, is thirty-five. In 503 there are thirty-three simple chromosomes plus a V-shaped multiple.

Figs. 27–29. Polar view, metaphase spermatogonial figures of individual No. 438.

Figs. 30–36. Polar view, metaphase spermatogonial figures of No. 503. The V-multiple chromosome is conspicuous on the periphery.

Fig. 37. First spermatocyte, late prophase showing the long V bi-tetrad of the one-V type.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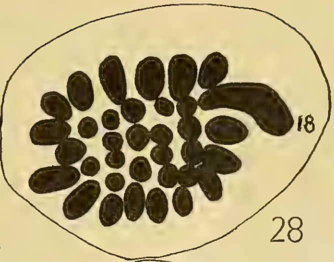
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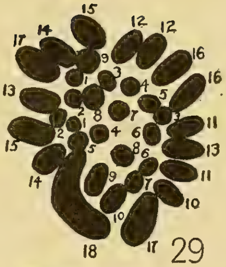
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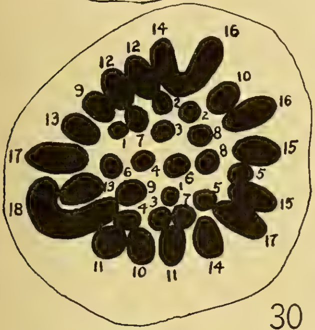
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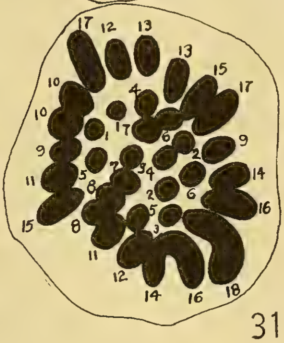
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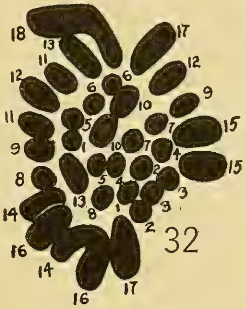
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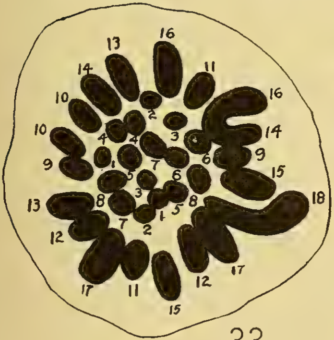
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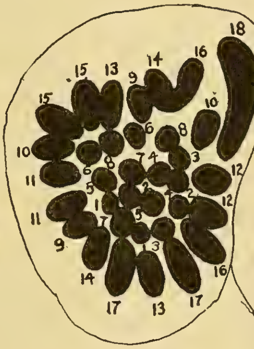
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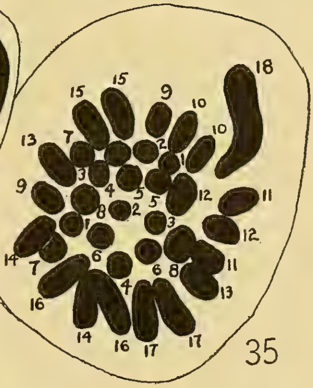
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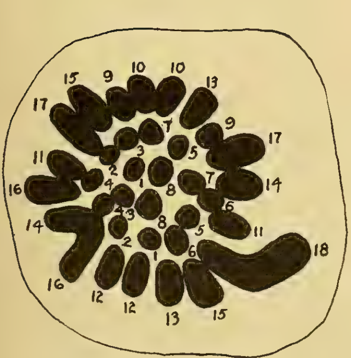
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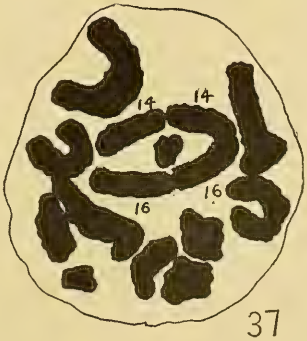
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Plate IV.

Jamaicana subguttata Walker.

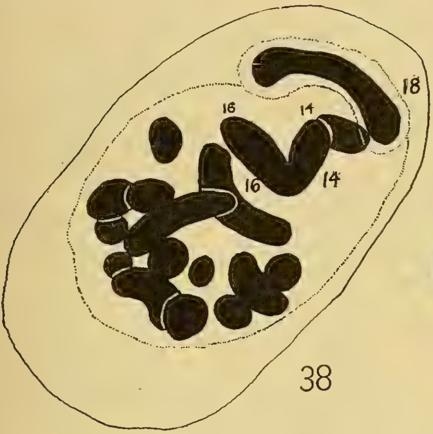
First spermatocyte figures of No. 503.

Fig. 38. Polar view prophase of first spermatocyte showing fifteen simple autosomes, the V-multiple, and the "accessory" chromosome. The latter is enclosed in a receptacle apart from the other chromosomes.

Figs. 39–41. Lateral view metaphase of first spermatocyte showing the multiple chromosome. The constrictions in the arms of the V show where the rod mates are about to pass to the opposite pole, breaking away from the multiple at the constricted places. The full number is not present but the accessory is prominent in 39 and 40.

Fig. 42. A spermatocyte figure showing the full number, 15, of tetrad chromosomes plus the double tetrad, plus the sex chromosome.

Fig. 43. First spermatocyte showing double tetrad and the accessory chromosome.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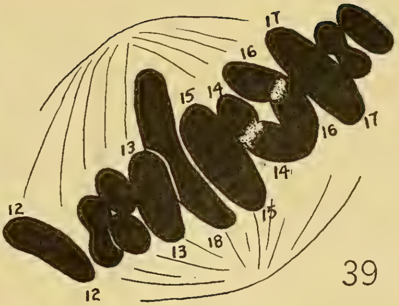
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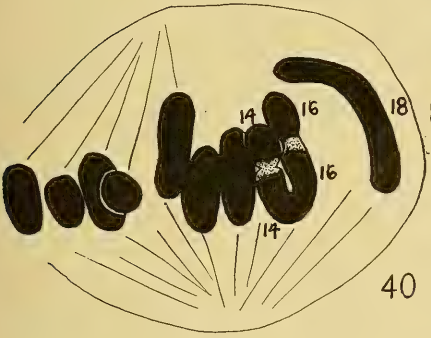
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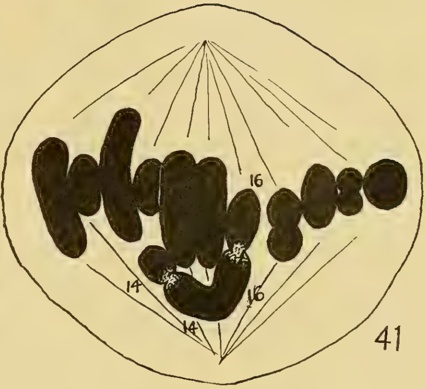

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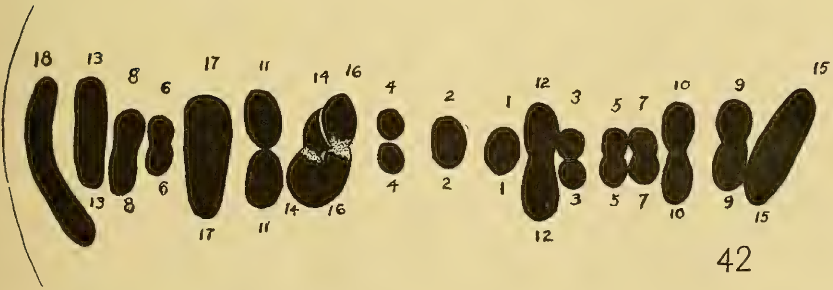
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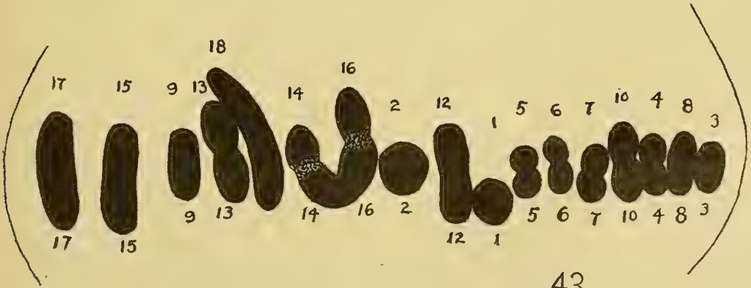
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C. I. WOOLSEY.

PLATE V.

Jamaicana subguttata Walker.

Spermatocyte figures of No. 503.

FIGS. 44–46. First spermatocyte anaphase. The multiple chromosome is to be seen in each figure. It has lost its rod mates, which have probably gone to the opposite pole, and is now the size of the spermatogonial V. By comparing it with the multiple before division as seen in Figs. 39–43, what has taken place, is more readily seen.

FIGS. 47. First spermatocyte telophase showing the V at one pole and its rod mates and the sex chromosome at the other. The latter is split longitudinally.

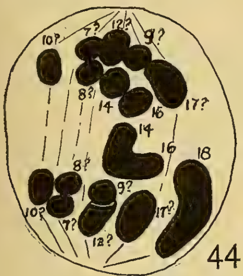
FIG. 48. Telophase of a first spermatocyte division.

FIG. 49. Telophase of first spermatocyte division containing the sex-chromosome and the multiple.

FIGS. 50–51. Telophase of the first spermatocyte or resting period of the second spermatocyte. The sex-chromosome is split preparatory to the second spermatocyte division.

FIG. 52. Second spermatocyte. Sex-chromosome divided. The V-chromosome is here divided and the arms are of the original size seen in the spermatogonial figures.

FIG. 53. Second spermatocyte. Sex-chromosome divided.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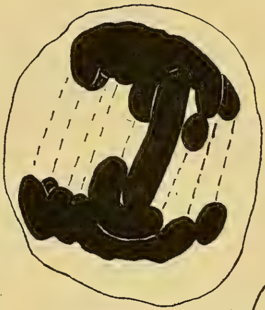

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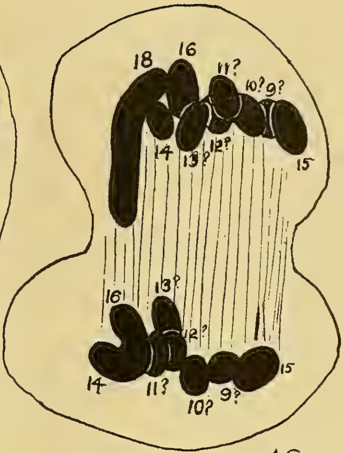
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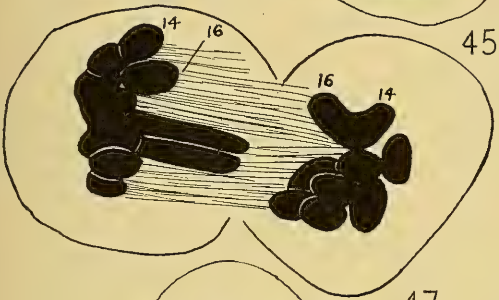

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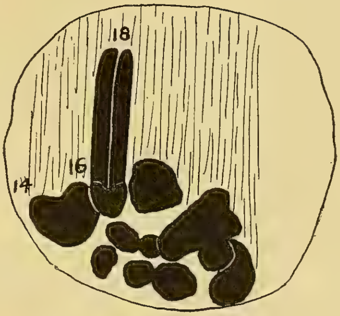

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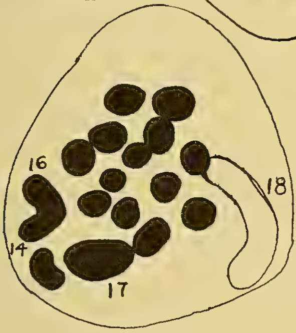

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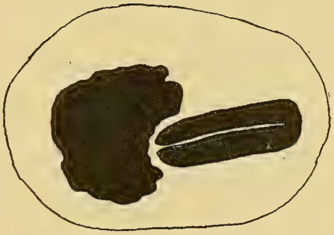

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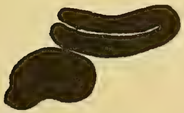

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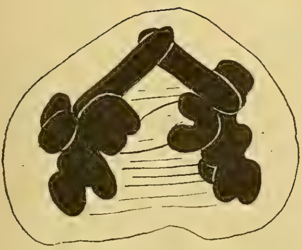

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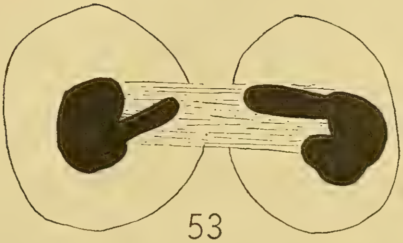

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C. . WOOLSEY.

PLATE VI.

Jamaicana unicolor n. sp. Caudell.

Spermatogonial figures of two members of this species. Thirty-five rods are found in the one; thirty-one rods and two V's are found in the other. Figures 65–67 are first spermatocytes.

FIGS. 54–57. Spermatogonial figures of individual 430. There are thirty-five chromosomes of the rod type here, paired and numbered according to size. No. 18 is the unpaired sex chromosome.

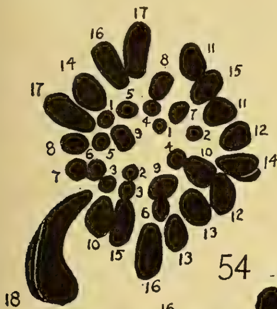
FIGS. 58–60. Spermatogonial figures of individual 560 showing thirty-one rods and two V's in each. No. 18 is the unpaired sex-chromosome. Just the largest chromosomes are paired and numbered.

FIGS. 61–64. Spermatogonial metaphases. Although the chromatin material is much massed, the sex and the two V-chromosomes are very prominent and distinct.

FIG. 65. First spermatocyte of individual 560 showing the multiple or bi-tetrad formed by the two V's.

FIG. 66. First spermatocyte prophase showing the complete number of chromosomes in individual 560,—fifteen rod tetrads, the bi-tetrad, and the sex-chromosome.

FIG. 67. The ring-shaped bi-tetrad more condensed in a latter prophase.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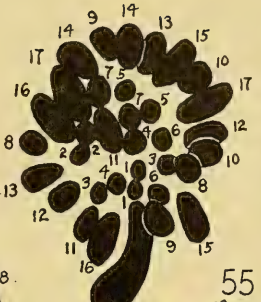
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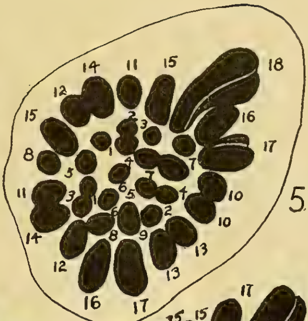
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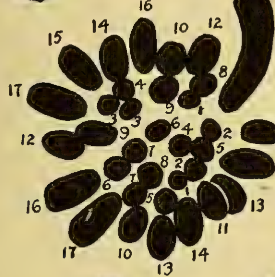
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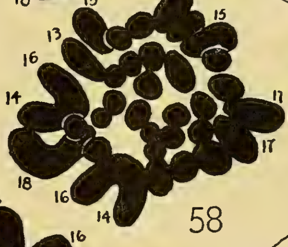
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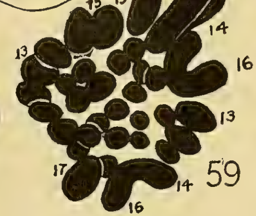
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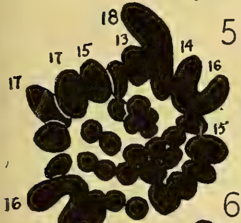
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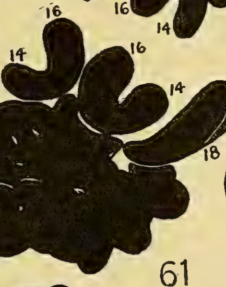
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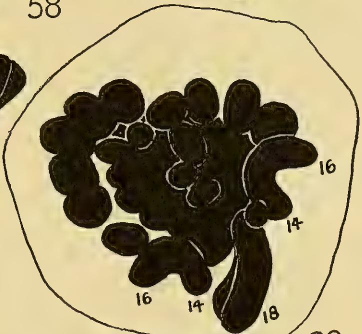
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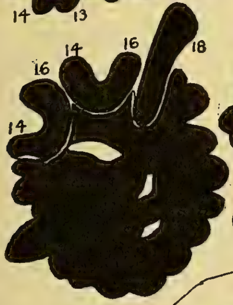
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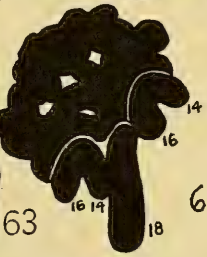
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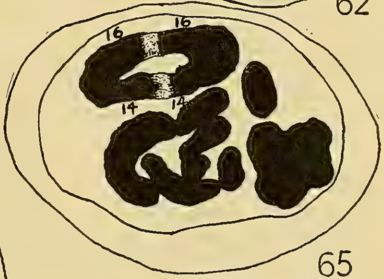

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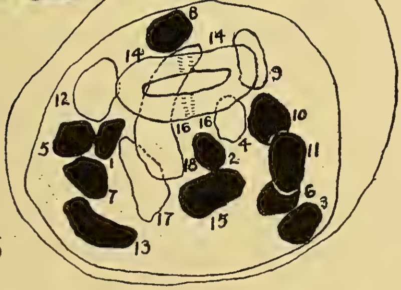
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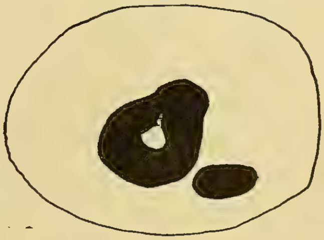

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C. I. WOOLSEY

PERIODICITY IN THE PRODUCTION OF MALES IN HYDATINA SENTA.[1]

A. FRANKLIN SHULL.

INTRODUCTION.

There is often a well-marked rhythm in the production of males in the rotifer *Hydatina senta*. Generation after generation may pass with few or no male-producing females; while later, in a few successive generations, male-producers may be abundant, only to be succeeded by a period in which male-producers are uncommon or wanting. Although this rhythm has not been mentioned by all students of the life cycle of *Hydatina*, and has been emphasized by few of them, it can hardly have escaped notice by any one who has bred this species for several months. In another genus, *Asplanchna*, Mitchell (1913) has laid stress upon this rhythmical appearance of males, as a basis for certain theoretical conclusions, and has called attention, by way of generalization, to the same rhythm in *Hydatina*.

Regarding the cause of this periodicity, there is not general agreement. Mitchell, writing of *Asplanchna* but extending his conclusions to rotifers in general, appears at times to regard the rhythm as the effect of an internal factor, and again as due to environmental conditions. He says "this rhythm is not the result of external conditions" but "is not absolutely independent of them."[2] Later he adds that "male production . . . is a matter of physiological potential and under the more or less direct control of nutrition" (p. 229). At other places he states that "male production . . . is a phenomenon all but wholly under nutritive control . . ." (p. 246); "these male and non-male producing strains . . . exist . . . and . . . these strains are also produced by nutritive changes" (p. 247); and "qualitative and quantitative changes in nutrition will be found the universal sex-controlling factors in this group" (the rotifers)

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[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

[2] *Op. cit.*, p. 228.

(p. 253). Whitney (1914), on the other hand, has no hesitancy in ascribing the periodicity of male production to external factors; thus, in mentioning earlier work of his own, "he was of the opinion that whatever the potent factor was that sometimes caused only females to be produced, and at other times caused nearly all males to be produced, it must be an external factor." On the basis of his recent work he attributes this periodicity to alternation of the active and quiescent states of the protozoan food of the rotifers.

In my own work on *Hydatina* during several years, certain lines have been bred so long, and so many families completely reared, that further light may be thrown upon the rhythm of male production. In the following pages evidence is first presented, bearing on the regularity of the periodicity of male production, and the probable independence of this periodicity of the environment. Later the supposed evidence that male production is correlated with nutrition is discussed.

Regularity of the Periodicity of Male Production.

Conditions Necessary to the Demonstration of Periodicity.

In parthenogenetic lines that produce many males, rhythm is not as easily demonstrated as in lines producing few males. If the proportion of males in a line producing many males be represented by a curve, there are so many irregularities in it, so many minor humps even in the periods of few males, that the larger humps are less striking. If the lines produce few males, on the other hand, the whole curve may be so lowered that the periods of depression are below the base line; that is, there are no males at all in these periods. In such cases the rhythm may be quite striking.

Further difficulty in detecting rhythm is introduced by rearing only one family in each generation. There are great individual differences between families taken at the same period, so that the family chosen may or may not be an average of all families that might be reared at the same time. Furthermore, the method of publishing the results, merely giving the total for the one family in each generation, often makes the rhythm appear less definite; because it often happens that in three or four successive generations, in a period of many males, the males are produced mostly by the daughters at the end of the family in the first of these generations, by the daughters in the middle of the family in the second generation, and by the daughters in the first part of the family in the third generation. In the manner in which these results have been published, the males of the first and third generation (appearing under date of the beginning of their respective families) seem to be four or five days apart, whereas they may be only one day apart, or even hatched on the same day.

It seemed advisable to obviate as many of these difficulties as possible, and to obtain evidence of periodicity less open to objection. The method adopted was as follows: First, a line was selected which was producing only a moderate number of males, with the expectation that the periods of male production would be completely separated by periods in which there were no males at all. Second, instead of isolating all the daughters of a single family in each genertion, parts of a number of families were isolated. This second precaution was taken to smooth out irregularities and to reduce the *apparent* length (and hence the overlapping) of the periods, both of many and of few males.

Lines Exhibiting Periodicity.

The line selected to meet the above conditions was one of the F_2 lines from a cross between an English and a Nebraska line, described in an earlier paper (Shull, '15). Several females of this line, all of approximately the same age, were placed together in a dish. When they reached maturity, the daughters of the early part of their families were rejected; but the daughters produced after about 24 hours of egg laying were preserved. From these daughters the "sex ratio" (ratio of male-producing to female-producing females) was determined, and from them also the next generation was reared. The first daughters were in each case rejected because, as I have shown before (Shull, '10), the first daughters are less commonly male-producers than are daughters in the middle of the family.

The daughters selected for rearing the next generation were in like manner kept together in one dish until about 24 hours after their first daughters appeared. At this time a second lot, all of about the same age, was isolated to determine the sex ratio of the second generation, and so on.

From 30 to 100 daughters, all of nearly the same age, and from the same parts of their respective families (end of first day of egg-laying) were recorded for each generation. A new generation was, by this method, secured every three days, as a rule, instead of every two days, as is possible when the first daughters of a family are used as parents of the next generation.

The number of male-producing and female-producing females in each generation thus recorded is given in Table I. Male production in this line, when bred as described above, showed a well-marked periodicity, the interval being about one month. Conditions were purposely kept fairly uniform; but even if it were not possible to prevent changes of the medium, it is scarcely probable that any external condition favoring male production should have recurred with such regularity. Moreover, were this periodicity due to external factors, the intervals between periods of male production should be the same in all lines bred at the same time and subjected to the same conditions. That this was not the case will now be shown.

TABLE I.

SHOWING THE NUMBER OF MALE-PRODUCING (♂ ♀) AND FEMALE-PRODUCING (♀ ♀) FEMALES IN A LINE OF *Hydatina senta*.

The male-producers occur periodically.

Date.	Number of ♂ ♀.	Number of ♀ ♀.	Date.	Number of ♂ ♀.	Number of ♀ ♀.
Jan 16	2	24	Mar. 14	0	44
19	0	24	17	0	38
22	1	35	20	0	42
25	3	33	23	0	23
28	19	46	26	8	40
30	14	46	29	36	48
Feb. 2	0	56	Apr. 1	7	71
5	0	40	4	1	51
9	2	38	7	0	37
12	0	43	10	0	40
15	0	47	13	1	36
18	0	48	16	0	35
21	0	13	19	0	43
24	0	35	22	3	85
27	17	21	26	68	51
Mar. 2	13	27	29	32	23
5	6	29	May 2	18	34
8	2	36	5	3	50
11	0	42	8	0	44

In another line, bred for a much longer time than was the line described above, there was equally clear evidence of regular periodicity, though the extent of the waves of male production was not determined. A line of rotifers obtained from England in the fall of 1912, described in another paper (Shull, '15), has been reared up to the present time. No considerable numbers of individuals of this line have as a rule been *isolated*, hence the sex ratio can not be stated; but during the time when the periodicity of other lines was being examined, several dozen individuals of most generations were reared in two or three dishes. As the number of males was always small in this line, males were found in these "mass" cultures only occasionally. They nearly always appeared in the dishes containing several successive generations, and then were wanting for a considerably longer period. There was thus a rhythm of male production, which, as shown in Table II., proved to be fairly regular and definite, though the number of male-producers in each period was not known.

TABLE II.

SHOWING DATES BETWEEN WHICH MALES WERE PRODUCED IN AN ENGLISH LINE OF *Hydatina senta*.

No males appeared between one period of male production and the next.

Number of Period of Male Production.	Dates Between Which Males Were Found.
First	February 13 to 25, 1913
Second	April 17 to 26, 1913
Third	June 19 to 24, 1913
Fourth	August 24 to 30, 1913
Fifth	October 31 to November 15, 1913

The interval between periods of male production was, in this line, a trifle over two months. Inasmuch as this line was bred in part simultaneously with the one recorded in Table I., the difference in the interval of male production in the two lines (one month in the first, over two months in the second) effectually disposes of any suspicion that this rhythm was induced by external conditions.

A third line which showed evidence of periodicity in the production of males was obtained from Nebraska in 1912, and has been reared ever since. This line is also described in a recent paper (Shull, '15). Complete families were isolated from early in November to December 15, 1912, but no males appeared. During the time when periodicity of male production was being studied, representative mass cultures were reared. These cultures showed males in small numbers only, and at times separated by wide intervals. The times of male production, as far as known, are shown in Table III., though the proportion of males was not recorded.

The periods of male production in the Nebraska line are separated by intervals of three to five months, the interval increasing with the age of the line. Though no complete families were reared, during any of these "waves" of male production, it was evident that in the later periods there were fewer males than in the earlier ones.[3]

Table III.

Showing Times at Which Males Were Produced in a Nebraska Line of *Hydatina senta.*

Number of Period of Male Production.	Dates Between Which Males were Found.
First	January 15 to February 1, 1913
Second	April 22, 23, 1913
Third	August 10 to 16, 1913
Fourth	December 7 to 11, 1913
Observation wanting[1]	
Sixth(?)[2]	November 1, 1914

In each of the three lines described, there was a well-marked rhythm in the production of males. The great regularity of this periodicity, especially in the first two of these lines, and the fact that the lines differed considerably from one another in regard to the interval between periods of male production, forbid the assumption that the waves of male production were brought on by specific external conditions.

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[1] Observations were wanting from March 8 to June 10, 1914. Probably not more than one period of male production fell between these dates, hence the next males recorded are to be regarded as belonging to the sixth period.

[2] A single male-producing female appeared in this "wave" of male production.

[3] In the English line described in Table II., while the intervals between periods of male production did not increase, with the age of the line, the number of males in successive periods plainly decreased. This is a confirmation of a conclusion which I formerly drew from lines bred through shorter periods, namely, that the proportion of male-producing females gradually decreases with the age of the line. Whether, as Mitchell suspects, this decrease is due to uniformity of conditions, is a question not answered by the evidence.

No statement here made is to be construed, however, as a contradiction of my former claim that external conditions may alter the *extent* of male production.[1] Few biological facts are more firmly established than that external factors modify the life cycle of *Hydatina*. The results described above merely show that, under fairly uniform conditions, there is nevertheless a periodicity in the production of males which must be due to internal factors.

Periodicity and Nutrition.

Mitchell ('13) has pointed out that in *Asplanchna* periods of male production are also often, perhaps usually, periods of vigorous growth and rapid reproduction; and he concludes therefrom that male production is a result of high nutrition. This conclusion may be correct, but it is scarcely logical, since coincident events are not always related to one another as cause and effect. But assuming as Mitchell does that size of family is a guide to nutrition, let us examine all the sources of information that are extensive enough to be of value, to determine, if possible in this way, the relation of nutrition to male production. In my own work in the past few years, there have been two lines in which hundreds of families have been reared. By collecting all of the families of the same size in a single line, and recording the proportion of male-producers, it should be possible to discover to what extent size of family and male production are correlated. Obviously one must not collect in the same group families belonging to two or more unrelated lines, for one of these lines may have larger families, and at the same time (but from other causes) either many or few male-producers, so that the groups of families of large size would have on the average a correspondingly high or low proportion of male-producers. Such an apparent correlation would have no significance. Within a single line, however, no such error could affect the results. The two tables herewith presented (IV. and V.) are each compiled from families belonging to a single line.

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[1] My discovery several years ago of internal differences between parthenogenetic lines of *Hydatina senta*, the result of which is a different proportion of male producers in each line, is characterized by Mitchell as a "return to the position of Punnett." Since Punnett never found an effect of external conditions, and since I never repudiated my experiments proving the effect of external conditions, there can have been no "return."

Notwithstanding the great fluctuation of the proportion of the male-producing females in families of different sizes, it might be possible to see in the percentages given in the last columns of these two tables (or perhaps only Table IV.), a slight increase from top to bottom, and hence a correlation between size of family and male production; though the degree of correlation certainly can not be high. However, even if such correlation exist, it does not follow that the percentage of male-producing females is dependent upon the degree of nutrition, which determines size of family. The one way to test the effect of nutrition is to alter it artificially, and note the results. That has been done in my starvation experiments (Shull, '10, pp. 320 ff.), with results that were positive but of such a character that they could be explained as due to changes in the chemical composition of the medium, rather than changes of nutrition. Mitchell ('13) objects that in drawing conclusions from these starved families, I have regarded only the totals; had I observed individual families, he believes, I would have reached a different result. Mitchell states that the male-producing females in my starvation experiments did not appear in the smallest families, which were presumably the offspring of the most starved parents, but in the larger families, produced by the better nourished females; and from this supposed fact concludes that abundant male production is due in *Hydatina*, as he believes it to be due in *Asplanchna*, to

TABLE IV.

SHOWING SIZE OF FAMILY AND PROPORTION OF MALE-PRODUCING (♂♀) AND FEMALE-PRODUCING (♀♀) FEMALES IN A SINGLE PARTHENOGENETIC LINE OF *Hydatina senta*.

Size of Family.	Number of Families.	Number of ♂♀.	Number of ♀♀.	Percentage of ♂♀.
1 to 5	7	3	13	18.7
6 to 10	15	15	96	13.5
11 to 15	16	47	158	22.9
16 to 20	20	28	322	8.0
21 to 25	20	73	388	15.8
26 to 30	21	152	437	25.8
31 to 35	33	174	909	16.1
36 to 40	27	213	816	20.7
41 to 45	29	281	977	22.3
46 to 50	26	324	931	25.8
51 to 55	19	285	710	28.6

high nutrition (coupled with irregularities of nutrition). How far his statement that the male-producing females are in the larger families is correct, may be seen from Table VI., in which

Table V.

Showing Size of Family and Proportion of Male-producing (♂♀) and Female-producing (♀♀) Females in a Single Line of *Hydatina senta*, Distinct from that in Table IV.

Size of Family.	Number of Families.	Number of ♂♀.	Number of ♀♀.	Percentage of ♂♀.
1 to 5	12	2	38	5.0
6 to 10	17	13	121	9.7
11 to 15	19	67	183	26.8
16 to 20	29	105	415	20.1
21 to 25	32	152	587	20.5
26 to 30	17	48	418	10.3
31 to 35	22	149	577	20.5
36 to 40	15	113	463	19.6
41 to 45	18	76	704	9.7
46 to 50	19	192	716	21.1
51 to 55	8	106	314	25.2

the families of the starved line in the experiment referred to (Shull, 1910, Table III.) are tabulated. The group of families containing 1 to 5 daughters, and that of families numbering 41 to 45, are omitted because there is but one family in each group.

Table VI.

Showing Size of Family and Proportion of Male-producing (♂♀) and Female-producing (♀♀) Females in a Starved Line of *Hydatina senta* Described in a Former Paper.

The greatest proportion of male-producers is in families of medium size.

Size of Family.	Number of Families.	Number of ♂♀.	Number of ♀♀.	Percentage of ♂♀.
6 to 10	7	7	49	12.5
11 to 15	3	6	34	15.0
16 to 20	6	39	66	37.1
21 to 25	8	88	101	46.5
26 to 30	16	172	273	38.6
31 to 35	8	96	169	36.2
36 to 40	2	14	64	17.9

In this table it appears that the greatest number of male-producing females is found, not in the largest families, but in those of medium size. It may also be recalled that the distribution of the male producers with regard to size of family, in these starved families where nutrition was *known* to have been variable, is not the same as the distribution in the well-fed families of Tables IV. and V., about whose nutritive conditions we know only that which size of family tells us. The argument that the numerous male-producing females of the starved line were produced as a result of high nutrition of their parents, loses much of its weight when it is shown that these male-producers were not chiefly in the largest families.

It is not to be asserted that nutrition has no effect upon male production. Indeed, Whitney ('14) has presented new evidence that *qualitative* differences of nutrition do affect male production. It is not clear what relation Whitney's results have to the question of *periodicity* of male production, whether changes of nutrition can be made to destroy the rhythm, or wholly to alter the interval, or merely to modify the extent of male production. My own starvation experiments, referred to above, left the intervals between the periods of male production unaltered, but the waves of male production and the intervening periods of female production were rendered less striking. I attributed the effects shown in these experiments to the chemical nature of the medium, and not to nutrition. Until experimental evidence indicates the contrary to be true, it is safest to assume that nutrition also, when it affects male production at all, does not alter the interval between periods of male production, but merely the extent of male production.

To summarize: Three lines of *Hydatina*, bred through many months, showed fairly regular periodicity in the production of males. One line exhibited relatively abundant male production every month; another every two months; while in the third the interval varied from three to five months during the period of observation. The fact that the interval between the periods of many males is quite regular in some lines, and is not the same in all lines reared simultaneously, indicates that this periodicity is due to an internal factor. Hundreds of families were examined to determine whether the largest families, which were presumably offspring of the best nourished parents, contained the greater number of male producing females, as Mitchell assumes they do. In well nourished lines there is some doubt whether there was any correlation between size of family and number of male producers; in starved families, on the other hand, the greatest numbers of male producers were not in the largest families, but in those of medium size. On the statistical evidence as a whole, the influence of quantity of nutrition upon male production is held to be "not proven." When qualitative differences in nutrition affect male production, the interval between periods of many males probably remains unchanged.

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NOTE ON TREMATODE SPOROCYSTS AND CERCARIÆ IN MARINE MOLLUSKS OF THE WOODS HOLE REGION.[1]

EDWIN LINTON.

In the summers of 1909 and 1910, while engaged in the study of the parasites of fishes at the laboratory of the Bureau of Fisheries, Woods Hole, Mass., I examined a number of invertebrates for larval stages of trematodes. The results of these examinations, although rather meagre with respect to the number of species found, are not without interest.

The following species of mollusks were examined: *Crepidula fornicata*, *C. plana*, *Ilyanassa obsoleta*, *Littorina littoria*, *L. rudis*, *Modiolus plicatulus*, *Mya arenaria*, *Mytilus edulis*, *Neverita duplicata*, *Pecten irradians*, *Purpura lapillus*, *Urosalpinx cineria*, *Venus mercenaria*. Besides these several species of crustaceans were examined, also one annelid, *Hydroides dianthus*.

Larval trematodes were found in only two species of mollusks, viz. *Ilyanassa obsoleta* and *Pecten irradians*. No trematode parasites were found in any of the crustaceans. A sporocyst found in the annelid, *Hydroides dianthus*, has already been reported.[2]

1. SPOROCYSTS AND CERCARIÆ FROM ILYANASSA OBSOLETA.

FIGURES 1–6.

Snails of this species were examined on six occasions. In all but one of these examinations sporocysts were found. On each occasion a considerable number of the snails were examined with much care, the several organs being teased under a lens. None of the sporocysts, however, were seen in place, in all cases having been found lying at the bottom of the dish in which the snails had been dissected.

The following extracts from my notes made at the time of collecting will give details of frequency of occurrence with such other observations as seem to be appropriate.

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[1] Published by permission of Commissioner of Fisheries.

[2] BIOLOGICAL BULLETIN.

1909. July 3, 34 snails examined, no parasites found.

July 19, 110 snails examined, 21 sporocysts found. The sporocysts were inactive and contained tailless cercariæ which were very active. So far as examined the sporocysts in this lot contained relatively few cercariæ, 9 being the greatest number seen, and as few as 2 noted in one sporocyst.

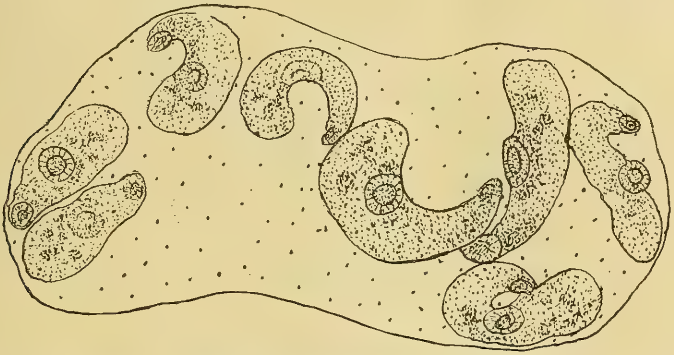

FIG. 1. Sporocyst containing cercariæ, from *Ilyanassa obsoleta;* in sea water flattened under cover glass. Length 0.86 millimeter.

Three sporocysts lying free in sea water had the following dimensions in millimeters:

Length	0.62	0.75	0.88
Breadth	0.30	0.26	0.30

Two cercariæ, killed under cover-glass over flame, had the following dimensions:

Length	0.31	0.42
Breadth, anterior	0.05	0.07
middle	0.13	0.14
posterior	0.06	0.06
Anterior sucker	0.04	0.05
Ventral sucker	0.07	0.06

July 20, 120 snails from North Falmouth. A dozen or more of these were dissected under a lens in the endeavor to find exactly where the sporocysts occur but without finding any in place. All the snails were then picked to pieces, washed, and the water decanted. About 100 sporocysts were obtained. These lay motionless on the bottom of the dish. They were easily seen on a black background, being whitish translucent. They were short oblong with rounded ends, often slightly arcuate.

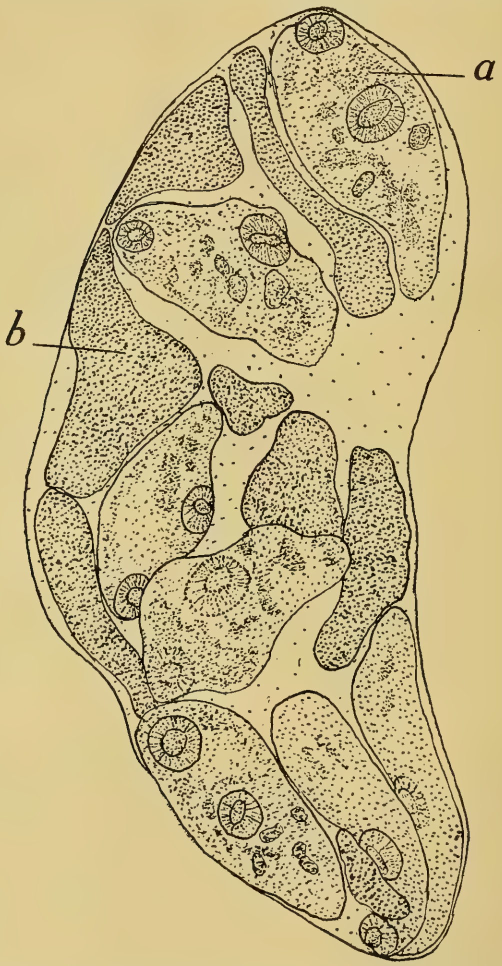

FIG. 2. Sporocyst from *Ilyanassa obsoleta*, flattened under cover glass, fixed over flame, stained and mounted in balsam. *a*, cercariæ. *b*, germinal cell masses and young stages of cercariæ. Length of sporocyst 0.80 millimeter.

July 22, about 300 snails from Tarpaulin Cove were broken open and about 80 of them removed from their shells. A few of these were looked over carefully, much of the material being teased and examined with the aid of the compound microscope. No sporocysts were seen in place. Sporocysts were found on the bottom of the dish in which the snails that had been removed from their shells were lying, also in the dish which held the snails still in the broken shells. It would appear that the sporocysts are rather loosely lodged in the mantle cavity, since they make their appearance when the broken shells and partly exposed animals are shaken about in the water. Some 600 sporocysts were obtained from this lot in a short time in this way. When the snails were picked to pieces, washed, and the water decanted, an increased number of sporocysts were obtained. The number of cercariæ in these sporocysts was greater than that recorded in the

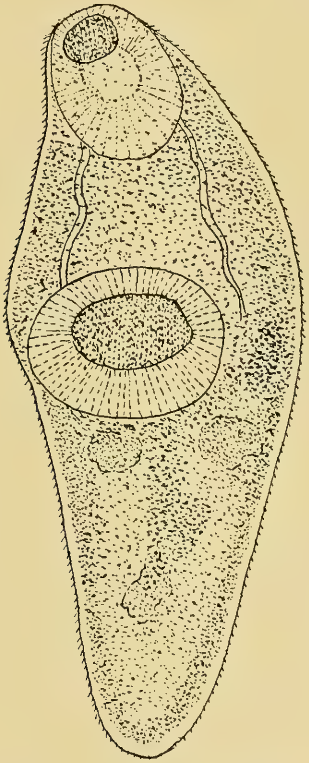

FIG. 3. Cercaria in sea water, ventral view, showing excretory vessels in front of ventral sucker, rudiments of testes, etc. Length 0.25 millimeter. From *Ilyanassa obsoleta*.

lot collected on July 19. One sporocyst when opened liberated 40 cercariæ. Some of these were immature. An anterior spine was noted for the first time on these cercariæ. It is embedded in the tissues of the head and may be seen protruding its sharp tip at the extreme anterior end in certain stages of extension while the cercaria is actively contracting. What were taken to be excretory vessels were seen extending from near the lateral margins of the oral sucker. They appear to unite in front of the oral sucker and again behind the ventral sucker. There was a large and conspicuous excretory space near the posterior end which communicated with the terminal pore by a slender canal.

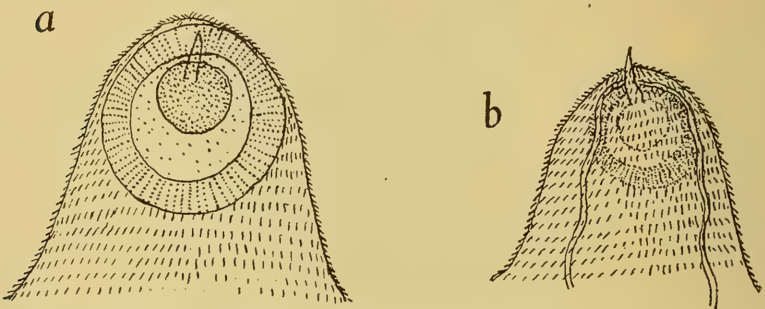

FIG. 4. Free-hand sketches of anterior end, life; showing oral spine, minute spines on surface of body, and anterior excretory vessels. *a*, dorsal view. Diameter of oral sucker 0.04 millimeter. *b*, Ventral view of another specimen. Diameter of oral sucker 0.06 millimeter; length of oral spine 0.017 millimeter. From *Illyanassa obsoleta*.

A cercaria, flattened slightly and fixed over the flame, had the following dimensions in millimeters: Length 0. 24, breadth 0. 10, oral sucker 0.041, ventral sucker 0.057, length of anterior spine 0.020. The posterior end was truncated as if slightly retracted.

July 23, 50 snails from a small salt water pond were removed from their shells and carefully dissected; about 350 others were broken open and stirred about vigorously. After a careful search 14 sporocysts were found. The pond from which these snails came, while salt, did not have free communication with the sea.

In 1910 a lot of snails that had been kept in a dish of sea water for several days were opened on different dates with the following results:

August 24, 24 snails were removed from their shells, picked to pieces with forceps, washed, the water decanted, and about 10 sporocysts found. These were linear oblong, frequently arcuate and slipper shaped, thickish. Dimensions in millimeters:

Length	0.66	0.56	0.60	0.56	0.70	0.55
Breadth	0.25	0.26	0.25	0.25	0.33	0.25

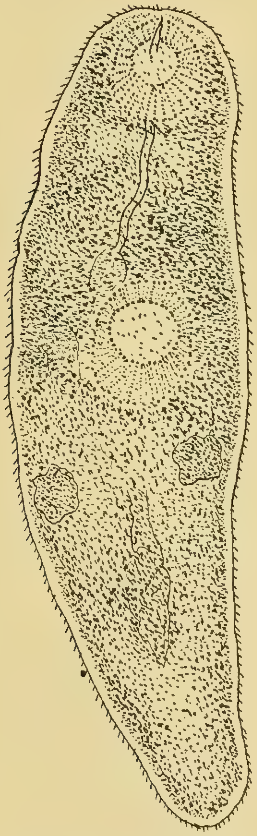

FIG. 5. Cercaria from *Ilyanassa obsoleta*, stained and mounted in balsam, dorsal view, showing oral spine, rudiments of prepharynx, pharynx, testes, etc. Length 0.30 millimeter.

August 26, 24 snails were examined that had been opened and removed from their shells the day before. No sporocysts were found. Another lot of 24 were opened and examined on this date. No sporocysts were found.

August 29, 36 snails examined in the usual way. No sporocysts were found.

The following notes were made on material that had been stained and mounted in balsam.

The shape of the cercariæ varies greatly but seems to be due to different methods of fixing, or, at least to different degrees of contraction. When fixed under pressure they are more or less

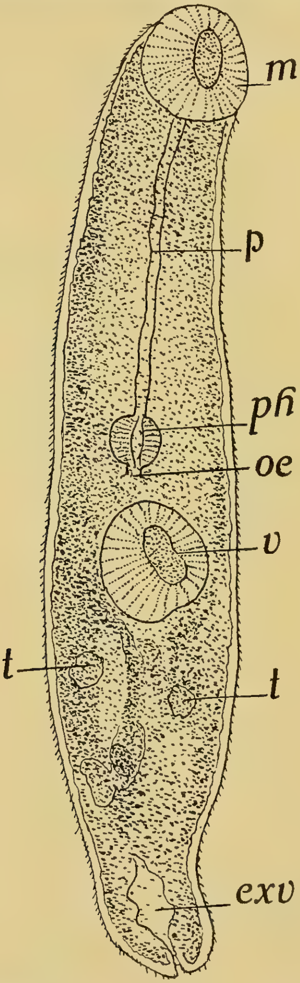

FIG. 6. Cercaria from *Ilyanassa obsoleta*, in balsam. Length 0.30 millimeter. *exv*, excretory vessel; *m*, oral sucker; *oe*, esophagus; *p*, prepharynx; *ph*, pharynx; *t*, testes; *v*, ventral sucker.

elongated. When much flattened they are usually long ovate, the greatest width being at the ventral sucker, which is near the middle of the length, thence they taper towards each extremity but more towards the posterior than the anterior end. When less compressed they may be long fusiform, or subcylindrical. The body is covered throughout with exceedingly minute spines. When the cercariæ are fixed without pressure they may be ovate, short fusiform, always thickish, frequently arcuate, the neck especially having a tendency to be bent ventrad. The suckers are nearly equal but there appears to be some variation. In most cases the ventral sucker is slightly larger than the oral. The apertures of the suckers are variable depending on the state of contraction when fixed. Frequently the aperture of the ventral sucker is transverse. In one case it was elongated axially. The aperture of the oral sucker was in most cases nearly circular. The pharynx is subglobular and lies near the anterior border of the ventral sucker. The intestinal rami were not distinctly shown. The anterior spine is not easily seen in the mounted specimens. The stained and mounted material does not usually show more of the excretory system than the posterior vessel which is very conspicuous in the living worms. In some of the mounted specimens this posterior vessel was evident; in others it could not be distinguished. In the flattened, oval individuals it was not seen (fig. 5). In the cylindrical forms it was usually visible (fig. 6). Rudiments of reproductive organs appear in all the older cercariæ. The most conspicuous of these are two lat-

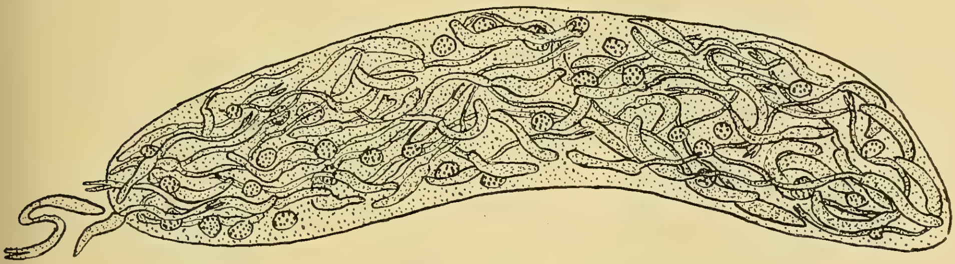

FIG. 7. Sporocyst with cercariæ, from *Pecten irradians*, life, under slight pressure; two cercariæ escaping from one end of sporocyst. Length 1.78 millimeter.

erally placed subglobular bodies which are situated a short distance behind the ventral sucker. These I take to represent the

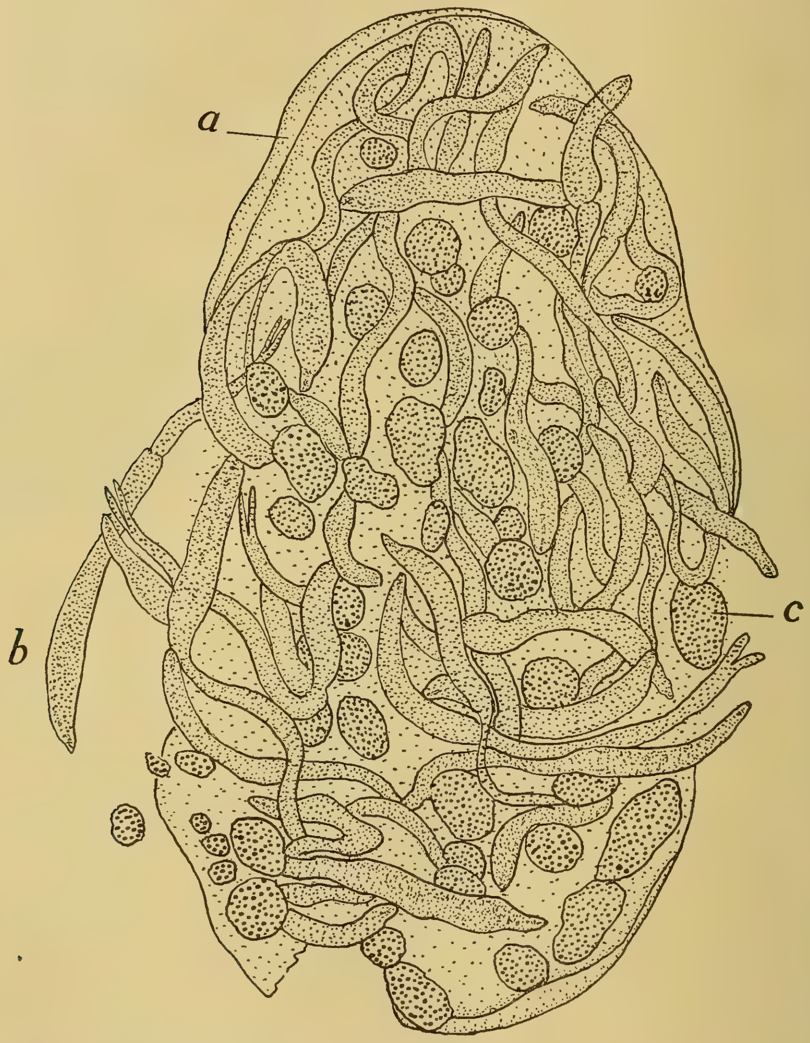

FIG. 8. Sporocyst with cercariæ, from *Pecten irradians*, stained and mounted in balsam; slightly crushed under the cover glass. *a*, wall of sporocyst; *b*, cercariæ; *c*, germinal cell masses and young stages of cercariæ. Length 0.60 millimeter.

testes. In front of the left testis may be seen, in some, a granular mass opposite the left posterior margin of the ventral sucker (figs. 3 and 5). This is probably the ovary. Behind the testes on the median line is a dense granular mass which is doubtless the beginning of the uterus. Granular masses which fill the body, but are most dense along the lateral margins may represent the beginnings of diffuse vitellaria.

The cercariæ of this species resemble *Cercaria linearis* Lespes, but the sporocysts are different.

2. Sporocysts and Cercariæ from Pecten Irradians. Figures 7–10.

In the summer or 1909 I examined 361 scallops on nine dates from July 3 to August 27 but found no sporocysts. In August, 1910, I examined 6 large scallops from Quisset Harbor. They had been kept in a vessel of sea water in the laboratory for two days before they were examined. After removing one valve the animals were shaken vigorously in sea water. A few small sporocysts were found in the bottom of the dish in which the scallops had been shaken. The scallops themselves were then examined carefully for sporocysts but no more were found. The sporocysts were elongate and slowly contractile with a tendency to become arcuate. The larger examples at rest in sea water measured 0.70 millimeter in length and 0.42 in breadth; length of one of the smaller specimens 0.30, breadth 0.15. A specimen compressed under a cover glass was 1.78 in length and 0.36 in breadth. These sporocysts contained numerous slender, tailed cercariæ. One of the latter in alcohol was 0.40 in length and 0.024 in breadth; another, length 0.20, breadth 0.027, length of body 0.085, length of tail 0.115. The first sporocyst examined had what appeared to be an actively contractile papilla at each extremity. These apparent papillæ proved to be cercariæ partially liberated from the sporocyst, but evidently held by the wall of the sporocyst contracting around them. All the cercariæ, both in the living and preserved specimens, are long and slender, the tail, in all cases, except immature specimens, being considerably longer than the body. In fully extended examples the tail may be two or three times as long as the body. When they are liberated from an active sporocyst they exhibit a peculiar jerking movement of the tail and posterior half of the body, the anterior end meanwhile being bent ventrad and performing a kind of pecking movement. This characteristic behavior of the anterior end is plainly in part due to the jerking movements of the posterior portions, and in part to the alternate protrusion and retraction of a short, proboscis-like organ at the anterior end. These movements suggest adaptations to enable the cercariæ to penetrate the soft membranes of the secondary host. In some freshly liberated individuals a thin, hyaline, membranous, fin-like border was distinguished. On one of these cercariæ this membrane was observed to be broken up into slender rod-like processes which resembled long cilia. The posterior extremity is divided into two slender branches. This forked extremity was also seen to be surrounded by a thin membrane in some fresh specimens. The structure of both body and tail is coarsely granular. Rudiments of what probably represent the oral sucker, and the pharynx were distinguished. When a sporocyst is crushed, there are seen, in addition to the cercariæ, of which there may be many stages of development, some granular material and balls of cells.

While the prevailing shape of these sporocysts is long and slender considerable variation exists. Both sporocysts and cercariæ are much like those found in the annelid *Hydroides dianthus*. The cercaria resembles *Cercaria cristata* La Valette.

3. Pearls.

While the examination of numerous specimens of the edible mussel (*Mytilus edulis*) resulted negatively, so far as trematode larvæ were concerned, a few cysts were noted, some of which may have been caused by trematodes. At my request Dr. Irving A. Field, who was opening large numbers of mussels in connection with his study of the development, and experimental work on the food value of this mollusk, handed to me those that in any way appeared to be abnormal. The number of such was small.

On July 24, 1909, 2 mussels were brought to me by Dr. Field, which he thought to be in poor condition. They had been cooked, so that there was no opportunity to examine them alive. One of them had about 15 cysts from 0.5 to 2 millimeters in diameter, along the edges of the mantle, and 4 on the foot, 1.5 to

2.5 millimeters in diameter. One of these cysts when crushed proved to be filled with small granular cells irregular in outline. Besides these, 3 small pearls were found in the mantle. There were a number of small white cysts embedded in the mantle of the other mussel. These contained pearls, 58 small pearls having been obtained from them. Some of them were multiple. They measured from 0.3 to 1.12 millimeters in diameter.

On August 11 some small cyst-like yellowish masses of similar appearance to those collected on the 24th, were found on the foot and mantle of a mussel. Their contents resembled leucocytes. A smear preparation revealed round cells of different sizes, the prevailing size being about 0.01 millimeter in diameter, with very strongly staining nuclei. A very careful examination of over 100 mussels made on different dates failed to yield any parasites.

It is perhaps worthy of note that the redia stage is omitted from the larval stages of trematode development which I have found in the invertebrates of the Woods Hole region.

Reference may here be made also to another abbreviated trematode life history in the case of the distome, *Parorchis avitus*, from the Herring Gull,[1] where miracidia, still within the ova in the later folds of the uterus, contained each a single well-developed redia.

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[1] *Proceedings of the U. S. National Museum*, 46: 551–555.

AN EXPERIMENTAL STUDY OF THE BEHAVIOR OF AMPHIPODS WITH RESPECT TO LIGHT INTENSITY, DIRECTION OF RAYS AND METABOLISM.

C. F. PHIPPS.

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I. INTRODUCTION.

The object of this study was to determine the effect of reagents and conditions affecting metabolism upon the reactions of amphipods to intensity and direction of light rays. The reagents and treatments used were potassium cyanide, chloretone, starvation and lowered oxygen content. All experiments were performed in the laboratory either upon amphipods just brought in, or upon those kept in captivity from one to ten weeks.

Three species of amphipods found in the vicinity of Chicago were used in the experiments, namely, a swift stream species, *Gammarus faciatus* (Say); a sluggish river and lake species, *Hyalella knickerbockeri* (Bate); and a pond species, *Eucrangonyx gracilis* (Smith).

(*A*) *Methods*.—In all the experiments a dark room was used. The special apparatus was a light grader, designed and first used by Yerkes ('02) and described with diagrams by Mast ('11, p. 61) and Shelford ('14). In the light grader the animals are kept during experiments in a small rectangular tank having glass sides. A false bottom in this tank allows running water to pass through and thus keep the water the animals are in at constant temperature. Midway between this tank and the nernst lamp in the grader there is a partition having a triangular aperture and this aperture is covered by a lens. By this means an intense field of light is made to fall upon one end of the small tank when the latter is placed at the focal point of the lens. This field of intensity shades off to darkness in the opposite end of the tank because of the triangular opening, thus making an intensity gradient. The light which passes through the glass sides of the small tank is reflected by mirrors to a dead black wall in another part of the grader.

When ready for the experiment the animals were placed in the small tank, usually three at a time, and allowed to remain in darkness for a short time to recover from the shock of handling. Then the light was flashed upon the tank and immediately the animals were released from the glass tube with which they had been confined. Every thirty seconds the relative positions of the animals in the tank were recorded. In part of the experiments 40 readings each were taken, the first 10 of which were discarded because of the excitement of the animals due to handling and to the flashing of the intense light upon them. In the remaining experiments 25 readings each, with the first five discarded, were found to be enough to give typical results.

In each set of experiments the tank occupied by the animals was placed in three different positions: position 1, at right angles to the direction of the light rays; position 2, at an angle of 45° to the direction of rays with the *dark* end nearer to the lamp; position 3, at an angle of 45° to the direction of rays with the *light* end nearer to the lamp. The animals were first released in the field of intense light and a series of readings taken; then the same animals were released in the dark end, and oftentimes also in the place of medium light, the readings being repeated in each case.

About 50 of the 257 experiments performed were eliminated because of avoidable errors in preliminary work. The animals were selected at random from the pan when experimented upon and the reactions under different conditions were compared.

When treating the animals with KCN and the other reagents they were kept in shallow glass dishes with a glass cover plate sealed on with vaseline to prevent evaporation. In the experiments with these animals the same solutions which they had been kept in were used in order to avoid the possibility of any stimulation which might occur by changing to tap water. The untreated amphipods were kept, and experimented on, in tap water. Filtered tap water was used in working with starved animals.

It was necessary, first, to establish a standard for the normal reactions of untreated amphipods, and then to compare with this any different reactions of treated amphipods. The series of experiments on untreated amphipods, by which the standard for normal reactions was established, form a good control series with which to compare the reactions of treated amphipods. Untreated amphipods were considered normal if they were negative to intense light when the tank was in position 1 or position 3. They were also considered normal if they were positive to intense light when the tank was in position 2. The reaction of the animals in position 2 of the tank shows that the direction of rays has a stronger influence than light intensity, for, though negative to intense light, in no case in any of the experiments with untreated animals with the tank in this position did the majority remain in the dark area. We may conclude from this that amphipods are negative to direction of rays. With treated amphipods these normal reactions were reversed in many experiments as will be shown later.

It was impossible to separate the influence of light intensity from that of direction of rays in cases where the tank was in position 3. Normally with the tank in this position both light intensity and direction of rays force the animals to the dark end. The percentage of those seeking the dark end when the tank was in this position was much greater than when the tank was at right angles to the rays, showing again the negative reaction to the direction of rays. The ray direction does not function when the tank is at right angles to it, except possibly to force the animals to the side of the tank farthest away from the lamp. Comparative figures which will be given later indicate at least that ray direction may force the animals to the side farthest from the lamp. Possibly the data as regards light intensity *vs.* ray direction has been over-emphasized.

We can base our conclusions definitely on the effect of the direction of rays only upon reactions with the tank set in position 2. The standard for this experimental work is based therefore both upon light intensity and direction of rays.

II. Responses of Untreated Amphipods to Light Intensity and to Direction of Rays.

Table I. shows the reactions of untreated amphipods when the tank was at right angles to the direction of light rays (position 1). Not only are the three species compared but also the stock kept in the laboratory several weeks is compared with that freshly brought in.

Table I.

Untreated Amphipods.

Experimental Tank at Right Angles to Direction of Rays (Position 1).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.			
			Readings.	% +.	% ∝.	% —.	Readings.	% +.	% ∝.	% —.
Gammarus	4 weeks	150	150	37	21	42		0	0	0
	2 days	120	60	27	19	54	60	54	26	20
Hyalella	4 weeks	130	130	16	7	77		0	0	0
	1 day	150	150	13	10	77		0	0	0
Eucrangonyx	6 weeks	150	150	13	11	76		0	0	0
	1 day	60	60	20	7	73		0	0	0

+ indicates a positive reaction, ∝ an indifferent reaction, — a negative reaction.

The strongest negative reaction to intensity is shown by the *Hyalella* and *Eucrangonyx* species, 73 to 77 per cent. This means that in the 150 half minute readings, *e. g.*, *Eucrangonyx* 6 weeks stock, only 13 per cent. of the animals were found in the area of intense light, 11 per cent. in the medium or dim light and 76 per cent. in the dark area. Although 13 per cent. were in the field of intense light at the time the readings were taken, yet, in most instances, the stay in that area was but momentary. If the animals in their "random movements" or "busy explorations" entered the field of intense light they were plainly stimulated and usually darted back quickly to the dark area.

The typical reaction for the species, negative to intense light, was that given by a majority of the untreated amphipods in the series of readings taken, and the group was considered to give reversed reactions only when a majority, in a series of readings, was found in the region of greatest light intensity. Such a reversal was found with one group of *Gammarus*, fresh stock, where in 60 readings the majority, 54 per cent., were found to be positive to intense light, while but 20 per cent. were negative, at the time of the readings. Another group of *Gammarus* fresh stock, however, was just as strongly negative to intensity. In this case of reversed reaction the animals remained in the intensity field much more constantly than did those of other groups which showed strong negative reactions. These few reversals with *Gammarus* may be due to the intense light, or to some factor not recognized. Mast ('11) has reported cases where long continued or increased light intensity has reversed the phototactic reactions of certain animals. The terrestrial form of amphipods, *Orchestia agilis*, is negatively phototactic when first exposed to light, but becomes positively phototactic with bright light, the stronger the light the quicker the reaction.

When the tank was placed in an oblique position so that it was at an angle of 45° to the direction of light rays with the dark end nearer the lamp (position 2), the majority of reactions in all the experiments performed were normal. As stated above, this normal reaction is based upon both light intensity and direction of rays, and as the direction of rays exerts a stronger influence than does intensity, the animals were forced to the light end of the tank, when the tank was in this position, and so appear positive to intense light. The percentages of normal reactions (positive readings), with the tank in position 2, are not so high as in position 1 (negative readings). This undoubtedly is due to the fact that the influences of intensity and direction of rays were working against each other when the tank was in position 2.

The position of the tank was again changed so that it was at an angle of 45° to the direction of rays with the light end nearer the lamp (position 3). In all except one series of readings, namely with *Gammarus* fresh stock, the large majority of reactions were normal, that is, negative to light intensity and to direction of rays. In this one case of reversed reaction with *Gammarus* the same animals were used as in Table I., where a reversed reaction is shown. With the tank in position 3 the percentages of normal readings were much higher than is true of the other positions of the tank. This is due to the combined action of intensity and ray direction in position 3, both together forcing more animals to the dark or negative end of the tank than does intensity alone when the tank is in position 1. The average percentage is 78⅓ negative reaction for position 3 of the tank and 66½ for position 1.

In the above three positions of the tank a larger percentage of animals freshly obtained was negative both to light intensity and to direction of rays, in all cases except one series of readings, than were the animals kept in the laboratory for several weeks. Some factor or factors associated with long captivity apparently had an effect in lessening the negative responses of the amphipods to intensity and to direction of rays. Possibly the metabolic rate was depressed by laboratory conditions causing a tendency to a reversal.

Table II. shows the percentage of experiments giving normal and reversed reactions with all untreated amphipods, both with the tank at right angles and at angles of 45° to the direction of rays.

TABLE II.

UNTREATED AMPHIPODS.

Experimental Tank at Right Angles and at Oblique Angles to Direction of Rays (Positions 1, 2 and 3).

	No. of Experiments Performed.	No. of Readings.	Normal Reactions, Per Cent. of Experiments.	Reversed Reactions, Per Cent. of Experiments.
Laboratory stock	33	1,174	100	0
Fresh stock	27	990	85.1	14.9
Totals	60	2,164	93.3	6.7

III. Responses of Treated Amphipods to Light Intensity and to Direction of Rays.

(*A*) *Potassium Cyanide.*—Only stock kept in the laboratory for some weeks was used in these experiments. Three different strengths of cyanide were tried, N/100,000, N/125,000, and N/150,000. Both reversed and normal responses occurred with all three. Probably the N/100,000 is not too strong for work with these animals and quicker results may be obtained with this strength. The animals were kept in the different solutions for varying lengths of time, one to nine days.

In Table III. the reactions of the three species are shown with the tank in position 2. In this case the *Hyalella* gave no majority of reversals in any series of readings, though the other two species showed strong reversals. Where the animals were exposed a longer time, as with *Eucrangonyx*, all the experiments gave a majority of reversed reactions. When the tank was in position 1 or 3 the results were very similar to those in position 2.

Table III.

Amphipods Treated with Potassium Cyanide.

Experimental Tank at an Angle of 45° to Direction of Rays with the Dark End Nearer the Lamp (Position 2).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.
			Readings	% +.	% ∝.	% —.	Readings.	% +.	% ∝.	% —.	
Gammarus...	6 weeks	320	270	61	27	12	50	0	43	57	1–3 days
Hyalella.....	2 weeks	180	180	73	5	22		0	0	0	2–3 "
Eucrangonyx.	9½ weeks	90		0	0	0	90	26	22	52	2–9 "

(*B*) *Chloretone.*—For these experiments a solution of 0.0025 per cent. was used, which was strong enough to give a very perceptible odor of chloretone. The animals were kept in this solution from 8 to 12 days before experimenting. This length of time exposed undoubtedly was a factor in causing many more reversed reactions than with other treatments.

When the tank was in position 1 the *Gammarus* showed a much larger per cent. of reversed than normal reactions, but the other two species were all normal in the majority of readings. In the other two positions of the tank all three species had strong reversals, and the *Hyalella* and *Eucrangonyx* gave no majority of normal reactions in any series of readings. See Table IV.

Table IV.

Amphipods Treated with Chloretone.

Experimental Tank at an Angle of 45° to Direction of Rays with the Light End Nearer the Lamp (Position 3).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.
			Readings.	% +.	% ∝.	% —.	Readings.	% +.	% ∝.	% —.	
Gammarus...	8–9 weeks	150	60	35	10	55	90	56	17	27	8–12 days
Hyalella......	4 "	60		0	0	0	60	95	2	3	9 "
Eucrangonyx.	1½ "	60		0	0	0	60	57	7	36	9 "

(*C*) *Starvation.*—Amphipods from each habitat were kept in filtered tap water and starved from 4 to 14 days before experimenting. Reversed reactions occurred with all three species, some after short treatment, others only after long treatment. Table V. shows reversed reactions with *Hyalella* and *Eucrangonyx* when the tank was in position 2. *Gammarus* gave reversed reactions with the tank in positions 1 and 3, but the majority were normal with the tank in position 2.

Table V.

Starvation Treatment.

Experimental Tank at an Angle of 45° to Direction of Rays with the Dark End Nearer the Lamp (Position 2).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.
			Readings.	% +.	% ∝.	% —.	Readings.	% +.	% ∝.	% —.	
Gammarus...	6½ weeks	60	60	56	19	25		0	0	0	4 days
Hyalella.....	7 "	120	60	71	5	24	60	38	21	41	6–9 "
Eucrangonyx.	1½–3 "	120	60	40	21	39	60	32	17	51	5–14 "

(*D*) *Low Oxygen Content.*—The oxygen content of tap water was reduced by using a machine, devised by Shelford and Allee ('13), for deaërating water by a process of heating and then cooling to the required temperature. The oxygen content was reduced in some experiments to as low as 0.79 c.c. per liter. The dish containing the amphipods was filled with this low oxygen water and the cover sealed down with vaseline. Amphipods began to die after an exposure of about one day to low oxygen content of from 0.79 c.c. to 1.51 c.c. per liter. Only *Gammarus* gave a majority of reversed reactions in any series of readings (see Table VI.), and this took place with the tank in positions 1 and 3. The other two species gave no majority of reversed reactions in any position of the tank.

TABLE VI.

LOW OXYGEN CONTENT.

Experimental Tank at Right Angles to Direction of Rays (Position 1).

Stock.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.	C.c. of O_2 per Liter.
		Readings.	% +.	% ∝.	% —.	Readings.	% +.	% ∝.	% —.		
Gammarus...	120	60	0	5	95	60	67	11	22	19 hrs.	0.79
Hyalella.....	60	60	34	18	48		0	0	0	17 "	1.51
Eucrangonyx.	60	60	23	18	59		0	0	0	22½ "	0.99

In Table VII. the percentage of experiments is given showing normal and reversed reactions with all treated amphipods, both with the tank at right angles and at angles of 45° to the direction of rays.

TABLE VII.

TREATED AMPHIPODS.

All Three Species. Tank in Positions 1, 2 and 3.

Treatment.	No. of Experiments Performed.	No. of Readings.	Normal Reactions, Per Cent. of Experiments.	Reversed Reactions, Per Cent. of Experiments.
Potassium cyanide......	54	1,520	72.3	27.7
Chloretone............	27	810	44.5	55.5
Starvation.............	29	900	69.0	31.0
Low oxygen...........	22	720	68.2	31.8

Table VIII. shows that the percentage of reversals in the three species is quite different under the same treatment. Also, in the same species, the percentage of reversals varies for each reagent used.

TABLE VIII.

TREATED AMPHIPODS.

Reversed Reactions only.

	Gammarus, Per Cent. of Experiments.	*Hyalella*, Per Cent. of Experiments.	*Eucrangonyx*, Per Cent. of Experiments.
Reversed reaction with KCN.........	39.3	5.5	33.3
" " " Chloretone....	60.0	66.6	33.3
" " " Starvation....	66.6	18.2	25.0
" " " Low Oxygen...	36.3	50.0	0.0

IV. Sides of the Experimental Tank, in Relation to the Lamp, Occupied by the Amphipods.

At each reading, when the position of the animals in relation to intensity was taken, their position in relation to the sides of the tank was also taken. The object was to determine, if possible, whether the direction of rays influenced the animals to seek the side of the tank farthest from the lamp.

The results are shown in Table IX. The readings have no reference to the dark end of the tank. In positions 1 and 2 the largest per cent. of the animals was found on the side of the tank farthest from the lamp, while in position 3 the majority were found on the side nearest the lamp. This is not conclusive, however, and these lateral positions in the tank may be due only to "random excursions" or "busy explorations" that Holmes ('01) speaks of as characteristic of active animals. Possibly the animals were reacting towards their own shadow, in positions 1 and 2, rather than to ray direction. It is interesting to note that there is very little difference in the results between treated and untreated amphipods.

In none of the experiments was there evidence of orientation either to light intensity or to direction of rays.

Table IX.

Tank at Right Angles to Direction of Rays (Position 1).

	Side Farthest from Lamp.	Median Position.	Side Nearest to Lamp.
Untreated Amphipods......	48.5%	11.3%	40.2%
Treated "	49.2	14.9	35.9

Tank at Angle of 45° to Direction of Rays with Dark End Nearer the Lamp (Position 2).

Untreated Amphipods......	52.9%	7.7%	39.4%
Treated "	49.2	12.6	38.2

Tank at Angle of 45° to Direction of Rays with Light End Nearer the Lamp (Position 3).

Untreated Amphipods......	35.7%	11.5%	52.8%
Treated "	37.9	11.7	50.4%

V. Conclusions and Discussion.

(*A*) *Summary.*—1. In an experimental tank set at right angles to the direction of light rays and graded from intense light to darkness, pond, stream and river amphipods, as a group, seek the dark area, therefore are negative to light intensity.

2. When the same tank is set obliquely, at an angle of 45°, to the direction of rays with the dark end nearer the lamp, the amphipods are forced to the light area, even though they are negative to intense light. The stimulus of the direction of rays, to which the amphipods react negatively, has a stronger effect than the stimulus of light intensity.

3. If the experimental tank is set at an angle of 45° to the direction of rays so that the light end is nearer the lamp, normal amphipods, as a group, seek the dark end. In this case the direction of rays exerts the same stimulating effect as light intensity in forcing the animals to the dark area. This again shows a negative reaction to intensity and to direction of rays. A larger percentage show negative reaction, with the tank in this position, than when the tank is at right angles to the light rays.

4. When treated with certain depressing agents many of these amphiphods become reversed in their reactions to light intensity and to direction of rays.

5. Freshly obtained amphipods give a larger percentage of negative reactions, both to intensity and direction of rays, than do amphipods which have been kept in the laboratory for some time.

6. In these experiments there is no evidence of orientation of amphipods either to light intensity or to direction of rays.

7. Changes in the metabolic processes (physiological states) of the amphipods were undoubtedly the cause of reversed reactions in this series of experiments.

Some of the above results have been obtained also by other investigators. Holmes ('01) says that all aquatic amphipods studied by him were negatively phototactic, although three species of land amphipods studied were positively phototactic. Loeb ('04), writing about experiments on *Gammarus pulex* and other animals, says that "whatever increases the activity tends to increase the positive reaction to light, while anything which tends to quiet the animals tends to make them negative." He adds also that the *Gammarus pulex*, which is negative to light, can be made positive by adding to the water a little carbon dioxid, hydrochloric, oxalic or acetic acid, ether, chloroform, paraldehyde, alcohol, esters and all ammonium salts. Boracic acid, according to Loeb, does not reverse these amphipods, but Jackson ('10), in repeating Loeb's experiments, but using *Hyalella knickerbockeri*, found that a saturated solution of boracic acid does cause a reversal, the same as the other reagents. Jackson also found that some other acids and some alkalies produce the same effect. These reversals took place, however, only when he dropped the animals into the solution, for when he put the animals into distilled water and gradually added the chemicals no reversals took place.

McCurdy ('13) says that "sunlight modifies the normal physiological changes taking place in protoplasm, checking some of the processes and probably accelerating the others. A starfish in the light moves to the shade because of disturbance by light of his metabolism." A part of this disturbance was due to there being "less CO_2 given off by the starfish when it was put in the sunlight."

(*B*) *Metabolism, Physiological States and Reactions.*—Before answers can be given to many questions that arise, much more work along these lines must be done. Other methods and treatments must be used, such as high oxygen content, caffein, acids, alkalies, carbon dioxid content, etc.

From the data obtained from this series of experiments it is evident that the responses of aquatic amphipods, like those of many other animals experimented upon, are related directly to the physiological state or condition of the animal. Anything which disturbs the rate of metabolism of the animal alters the response to stimuli. Allee ('12) with isopods, Child ('10) with planaria, Wodsedalek ('11) with may-fly nymphs, and other investigators have found this to be true.

In these experiments on amphipods then, the reversed reactions are caused by some change in the metabolic processes of the animals. Potassium cyanide depresses the metabolic processes by decreasing oxidation. Oxidation is decreased by decreasing the ability of the tissues to take up oxygen. Chloretone is a soporific and has a depressing or inhibiting effect upon certain metabolic processes. Starvation decreases metabolism by removing the material to be oxidized. Such reagents and treatments are known to have specific effects on metabolism and they also cause reversals in phototaxis, therefore the responses are related to the metabolic rate of the animal.

Jennings ('04) says that physiological states are the most important determining factors in reaction and behavior. By physiological states he means the varying physiological conditions as distinguished from permanent anatomical conditions. Can we be sure that such physiological states do exist? If we subject animals to the same external conditions and give the same stimulus, and the animals react differently, then the difference must be due to variations in internal conditions; else the reactions would always be the same. A stimulus changes the physiological state of the animal as a whole, and this change in physiological state induces a certain type of reaction.

My thanks are due to Dr. V. E. Shelford under whom this work was done, and to Dr. W. C. Allee and Mr. M. M. Wells for valuable suggestions.

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A CASE OF PERSISTENT MELANISM.

H. E. EWING.

The occurrence of melanism is a phenomenon of wide distribution in nature, being recorded among animals belonging to a great many classes and orders. Our records show that while the occurrences are frequent and are found in species belonging to many of the larger zoölogical groups, yet the actual numbers of melanic individuals found among the individuals of any one species, in any one region is usually extremely small in comparison with the total number of normally colored individuals of the same species found in the same region. Because of this rarity of these black-colored individuals the appearance of melanic forms has been very generally regarded as being due to sporadic though at times oft-repeated, sporting. Such melanic forms do not usually persist racially. It was Darwin who years ago noted that sports of almost all kinds were ruthlessly eliminated in the struggle for existence; and sporting in the form of melanism apparently has offered no exception to this general rule.

Perhaps one of the best examples of the racial persistence of melanism is that of the melanic form of the moth, *Amphidasys betularia*, which existed as a rarity in England some years ago, but which now has replaced the typical form about some of the manufacturing districts. This persistence of melanism has been explained on account of the environment in these districts being changed by the smoke from factories which darkens the vegetation in general, by the killing of lichens and by the depositing of black soot, and in this manner gives an advantage to the melanic forms by making them less conspicuous than the normally colored individuals.

THE APPEARANCE OF MELANIC ROSE CURCULIOS.

During the summer of 1913, while in the Willamette Valley in western Oregon, I came across instances of melanism among individuals of one of our common beetles that were quite striking. We have in the Willamette Valley, as in almost all other sections of the country, the well-known rose curculio, *Rhynchites bicolor* Fab., which feeds chiefly upon the buds and flowers of wild and of cultivated roses. It will feed, however, upon a few other plants, especially the buds of wild blackberries, which grow so abundantly along the streams in western Oregon.

This weevil is about one fourth of an inch long, and in all the sections of the country where it has been observed outside of the Willamette Valley is of a red color above; while the underparts of the body, and sometimes the head and beak, are black. The red in some instances extends forward so as to include the head and beak. When viewed feeding on the roses, the dark under surface of the body is largely concealed so that the weevil appears almost entirely red.

The red color of the dorsal surface is possibly protective to the species when it is feeding on the petals of wild or cultivated roses, as the color harmonizes with the red of many roses, and for this reason might make the individuals much less conspicuous objects to hungry birds and other enemies.

I found feeding along with the red individuals of *Rhynchites bicolor* Fab. individuals which were totally black. At first I suspected that these black individuals were of a different species. Upon looking the matter up I found that they had in a few instances been collected, and were called *Rhynchites æneus* Fab., a black species, which is common in the eastern part of the country.

During the summer of 1913 I demonstrated that the two forms would breed together in captivity. However, of the several larvæ that I obtained from the eggs deposited none reached maturity. Following these experiments I made a critical study of the characters of our two forms found in Oregon, and failed to observe any differences in structure whatever, hence considered the black ones only as melanic individuals of the common rose curculio. In order to get the opinion of a specialist in Coleoptera, I showed specimens of the two forms to Dr. Van Dyke, of the University of California. He stated that the two were the same, and that the black form found in Oregon was not the *Rhynchites* *æneus* Boh. of the eastern states. I may here add that the real *Rhynchites æneus* Boh. is a very hirsute species; it has well-developed punctate striations on the elytra, and prominent marginal elytral grooves. Our black Oregon form has none of these characters pronounced,—the hairs are very small, the punctations in the elytral striæ are almost obsolete, and the marginal grooves of the elytra can hardly be noticed.

Mating and Other Habits.

During the spring and summer of 1914 I made many field notes on these two forms of *Rhynchites*. I found that the two were constantly associated. They fed together in the same way on the same rose bushes and even on the same buds. They were found to feed together on wild blackberries; they emerged from winter quarters at the same time, and, finally, they were repeatedly found to be interbreeding in nature.

Attempts at Rearing Hybrids.

Four of the individuals of the black form which were found mating in nature with red individuals were confined with their red mates in separate breeding cages. Here they continued to breed for over a month, after which they began to die. In the meantime the females had laid large numbers of eggs; but out of this large number, including several score, I am very sorry to state that I did not succeed in rearing a single individual to maturity. The whole trouble this year was that the buds of the roses soon died after they were punctured by the female, and fell to the ground, taking with them the developing larvæ. In these dead, shriveled rose-buds the larvæ invariably died. Even their transference to fresh rose-buds was of no use, for they would not stay in these buds when placed there artificially. None of these larvæ pupated.

It may be well to state that in all the literature which I have examined I have failed to find a single record of this weevil being reared from the egg to its adult state. Its life history has not been worked out by complete breeding experiments, although repeated field observations, together with fragmentary notes on the life history, have shown that it produces but a single brood a year and hibernates in the adult stage.

MATING STATISTICS.

Having been foiled in my attempts at breeding these beetles, I decided to attack the problem from a different standpoint, namely, to observe the mating habits in nature, to see if the black individuals mated as freely with the red ones as with those of their own color. During the months of May and June I made fourteen different observational trips to some patches of wild roses at the west end of the town of Corvallis, Oregon. I collected every pair that was observed mating, as well as those that were not observed mating; these were collected so as to obtain population statistics. The results obtained were as follows:

Total number of matings observed	53
Normal *Rhynchites bicolor* × normal *Rhynchites bicolor*	44
Melanic form × melanic form	0
Normal *R. bicolor* ♂ × melanic form ♀	3
Melanic ♂ × normal *R. bicolor* ♀	9

Thus we find that out of the total number of matings observed in nature, 83 per cent. were between normally colored individuals, while 16 per cent. were between melanic individuals and normal ones; no matings being observed between two melanic forms.

Although we can not prove anything in regard to the exact status of these two forms of *Rhynchites* from these observations alone, yet we may speculate, somewhat, in regard to a few points. They tend to indicate:

1. That the normal intergeneration of these two forms is followed by the segregation of the characters following the dominance in the first cross.

2. That the black form is recessive to the red.

3. That racial characters have become fixed without natural barriers, without isolation of any kind or without change of habit but purely through the segregation of the characters in the germ plasm.

POPULATION STATISTICS.

The population statistics obtained for the same patches of wild roses that the mating statistics were obtained from, and at the same time are as follows:

Rhynchites bicolor Fab., normal	588
Black form	68
Total for both forms	656

Thus out of the total of 656 individuals counted, 68, or 11 per cent. were melanic. This percentage is so large that I think that no one would suspect the occurrence of melanism in this species to be due to the sudden sporting of a great many individuals, but rather to the persistence of melanism through segregate inheritance of the melanic types from a few or even a single sport progenitor. In other words either melanism or other characteristics associated with it in this instance have been advantageous to the black forms under the particular conditions of the Willamette Valley to such an extent that a black or melanic race has been evolved in direct competition with the normal type of the species from which it sprang, and has not been ruthlessly eliminated as a race as is usually the case with melanic forms.

The future of this black race of rose curculios will be interesting to watch. I hope to be able soon to breed these forms successfully, and also hope to be able to ascertain what the conditions are in the Willamette Valley which apparently make melanism advantageous to this curculio. A description of this melanic form is here given. Even in the Willamette Valley its distribution differs from that of the normal *R. bicolor*.

Rhynchites pullatus new variety, or species.

All the individuals as yet observed are black throughout. Body and appendages clothed with very fine short hairs which are not noticeable with the naked eye. Snout subequal to the tibia of the first pair of legs in length, bent almost straight downward in the male, but extending forward so as to be plainly visible from above in the case of the female. The antennæ arise from a position a little in front of the middle of the snout in the male and a little behind the middle of the snout in the female. Antennæ well clothed with hairs; three distal segments much broader than the others and forming a club. Dorsal surface of thorax punctate. Around the margins of each elytron is a distinct groove. The longitudinal punctate lines are rather indistinct, and may be wanting. Femora of anterior legs somewhat swollen. Length of body excluding snout, 5.5–7.0 mm.

STUDIES OF FERTILIZATION.

VII. Analysis of Variations in the Fertilizing Power of Sperm Suspensions of Arbacia.

FRANK R. LILLIE.

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I. Introduction.

In his epoch-making "Expériences pour servir a l'histoire de la génération des animaux et des plantes" published in 1785 the Abbé Spallanzani describes among his numerous experiments on fertilization and artificial parthenogenesis some determinations concerning the minimal quantity of sperm necessary to fertilize the eggs of the frog. He found that he could get perfect fertilization with seminal fluid diluted 2,720 times with water. At greater dilutions the percentage of fertilized eggs began to fall off, but some eggs fertilized up to a dilution of about 20,000 times. He calculated that the weight of the "spermatic particles" necessary to fertilize an egg was 1/2,994,687,500 of a grain, and that the volume of the egg in proportion to the volume of spermatic particles necessary to fertilize it is as 1,064,777,777 : 1. In 1824 Prevost et Dumas confirmed these calculations.

So far as I know such experiments have not been repeated. The reason for this would appear to be that when it was once established that only a single spermatozoön unites with each ovum in fertilization all such quantitative studies of the fertilizing power of sperm dilutions appeared to have lost their point. So long as it was assumed (as was generally the case) that the fertilizing power of the spermatozoön is a function of its motility alone, that is, of its capacity to "penetrate the ovum," there could be no object in quantitative studies. But as it came to be recognized that the fertilizing power of the spermatozoön is associated with some definite substance that it bears, possibly either a lysin (Loeb) or an activator (self), the problem assumes a different aspect; for it is obvious that if the sperm should lose such a substance in any way, its fertilizing power would be lost even though its motility should be preserved unimpaired. In such a case the relative fertilizing power of sperm suspensions could not be measured either in terms of concentration or of activity of the spermatozoa. Variations in the fertilizing power of suspensions of known concentrations might, therefore, be a measure of the loss of the postulated fertilizing substance. On reflection it is obvious that the spermatic substance in question must be loosely bound to the sperm, because it exerts its first effect, that of inducing cortical changes in the egg, before penetration, as I have shown for *Nereis*, and Loeb for certain hybrid combinations; at this time, therefore, the spermatozoön must set free its receptors[1] (activators).

Recently Glaser (1913 and 1915) has maintained that in *Arbacia* more than one spermatozoön is needed for fertilization of the egg, even though only one actually penetrates. The observations on which this conclusion rests are no doubt correct, *under the given conditions*, and I have made similar observations, as will appear in the course of the present paper. But it by no means follows from the observations that a single spermatozoön may not be adequate under other conditions (and this can be demonstrated). We cannot, however, deny *a priori* the possibility that for the initial phases of fertilization a number of spermatozoa may be of assistance though only one enters and is concerned in later phases. If the phenomena of fertilization are to receive a physiological, and ultimately a chemical, interpretation, quantitative questions may be of serious significance.

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[1] In study VI. (1914), I propounded a theory of fertilization according to which the initiation of development of the egg is due to activation of an ovogenous substance, which I named fertilizin, contained in the cortex of the egg. In fertilization such activation is caused by a certain constituent of the sperm, which I called the sperm receptors; and the action of the fertilizin thus aroused must be on certain substances of the egg which I named in general egg-receptors. From a chemical point of view therefore we must have an interaction of three substances (or groups of substances), viz., sperm receptors, fertilizin, and egg receptors.

It would seem to be a perfectly simple matter to determine the greatest dilution of sperm at which any fertilization takes place, and to express in the form of a curve, from percentages of eggs fertilized, the rate of loss of fertilizing power due to dilution. This was the very simple problem with which the present investigation began. However the results were in the highest degree contradictory; the same lot of sperm might vary in a period of half an hour from 1/1,024 to 1/9,000,000 (or less) of 1 per cent. dilution in its power to fertilize the same percentage of a single lot of eggs. The investigation, therefore, turned to the problem of such variations and their cause.

II. Experiments.

1. *Methods.*

Quantitative methods cannot possibly be as rigorous in a problem of this kind as in a purely chemical problem. In the first place we have to deal with variable reagents in the ova and sperm of *Arbacia;* and in the second place the initial measurements must be made rather hurriedly, so as to ensure freshness of the reagents, and under conditions that do not injure their vitality; the available quantities of material also limit the methods of measurement.

Sperm.—The standard for measurements of sperm dilutions is the "dry sperm"; *i. e.*, the thick creamy mass that exudes from ripe testes of *Arbacia.* If a ripe male be opened and inverted in a dry Syracuse watch crystal a certain amount entirely free from foreign admixture usually flows from the genital pores and collects in a mass in the crystal. While this may in certain cases be as much as 2 c.c., usually it is a much smaller quantity. It is quite impracticable to measure this by graduated pipettes; I have therefore used a drop of this dry sperm from bulb pipettes of fairly uniform openings as a standard, and, reckoning 30 such drops to the cubic centimeter, have made "1 per cent. sperm suspensions" by the addition of such a drop to 3.3 c.c. of sea water. This is the standard suspension from which most of the experiments proceed, and all sperm suspensions are expressed in fractions of such a 1 per cent. suspension. Given perfectly dry sperm, the initial variation due to the method cannot be very great in relation to the tremendous range of variation in fertilizing capacity of sperm due to other causes. Indeed it is a vanishing quantity.

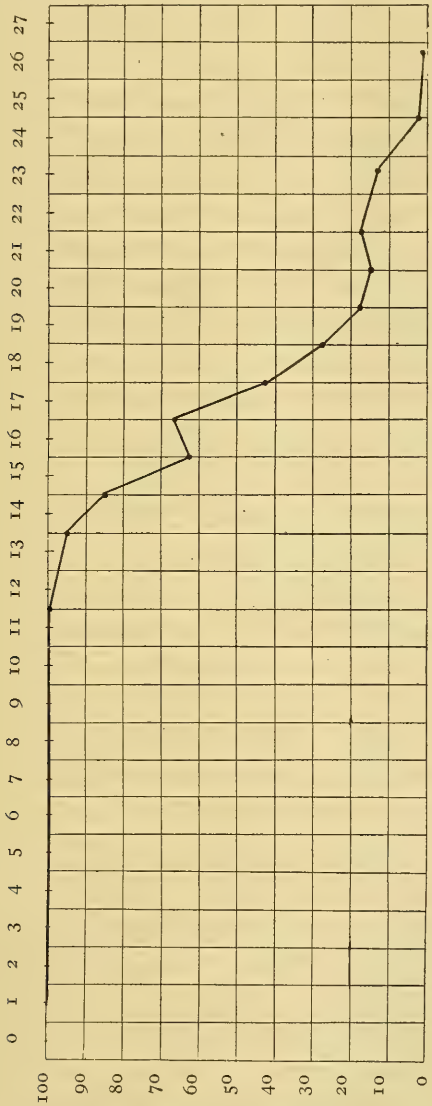
Eggs.—Egg concentration is a factor of relatively slight significance within the limits of the experiments, as will appear from the facts to be presented. Within a very wide range it does not affect the result measured in percentage of fertilized eggs. It is measured roughly by allowing washed eggs to settle for half an hour in a 100-c.c. graduated cylinder, and expressing the quantity there settled as a percentage of the entire fluid. There is of course for every concentration of sperm an egg concentration that is above the optimum for percentage of fertilization. But, as will be seen from the tables, such egg concentration lies beyond the concentration used in most experiments.

In most cases segmentation of the eggs was used as the criterion of fertilization, but membrane formation was also used in some cases, especially in high concentrations of sperm where many eggs failed to segment owing to polyspermy.

Formulation of Results.—The fertilizing power of sperm suspensions is expressed in curves whose ordinates are percentages of fertilization, and the abscissæ a geometrical series of dilutions of 1 per cent. sperm in powers of 2. This method was adopted for the abscissæ because of the method of successive half dilutions used in many experiments, and because the enormous range of fertilizing power made it impossible to compare results on one scale with an arithmetical progression. When it is realized that the fertilizing power may cease at 1/156 of 1 per cent., or extend to 1/90,000,000 the necessity of the geometrical series in the abscissæ will become apparent.

2. *The Optimum Curve of Dilutions.*

We may begin with the optimum curve of dilutions (Curve 1), because this answers most completely, and probably fully, to the current expectation that a single spermatozoön suffices for the fertilization of an egg. This curve is prepared from data of experiments calculated to bring eggs and sperm together in the freshest possible condition of the sperm. In general measured quantities of washed eggs were put in measured amounts of sea-water, and measured quantities of definitely calibrated sperm suspensions added and stirred in as uniformly as possible.



Curve I.

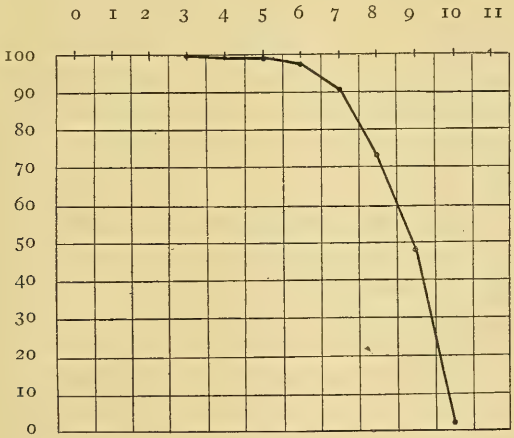
A control of unfertilized eggs in sea-water was always kept to guard against chance fertilizations. To illustrate: the last four determinations of the curve were made as follows: In four crystallization dishes were placed 1,000 c.c. sea-water (*A*), 3,000 c.c. sea-water (*B*), 1,000 c.c. sea-water (*C*), 3,000 c.c. sea-water (*D*). To each was added 2 c.c. of a washed egg-suspension (about 3 per cent. to 5 per cent.). The sperm was then prepared as follows: (1) one drop dry sperm to 3.3 c.c. sea-water at 9.43 A.M. = 1 per cent.; (2) 1 c.c. of sperm 1 to 99 c.c. sea-water 9.43.30 A.M. = 1/100 per cent.; (3) 1 c.c. sperm 1 to 999 c.c. sea-water 9.45.30 = 1/1000 per cent. To *A* was added 1 drop sperm 2(1/100 per cent.) at 9.43.45; to *B* one drop sperm 2(1/100 per cent.) 9.44; to *C* one drop sperm 3(1/1000 per cent.) 9.45.45; to *D* one drop sperm 3(1/1000 per cent.) 9.45.45. An assistant stirred in the sperm thoroughly as added. The sperm concentration in *A* was therefore 1/100 × 1/30 × 1/1000 = 1/3,000,000 per cent.; in *B* it was 1/9,000,000 per cent.; in *C* 1/30,000,000 per cent.; in *D* 1/90,000,000 per cent. 1/3,000,000 per cent. falls between 21 and 22 on the scale, and the others as shown. The exact times of mixing the sperm are given because, as will appear beyond, time is an extremely important factor with reference to fertilizing power.

To appreciate the extent of this dilution it may be said that beyond a dilution of 1/10,000 per cent. (between 13 and 14 on the scale) one can rarely find a single spermatozoön in the jelly of the fertilized eggs. At about 1/2000 per cent. (11 on the scale) the sperm suspension does not even appear opalescent. We may therefore feel reasonably sure that beyond about 14 or 15 on the scale a single spermatozoön certainly suffices to completely fertilize an egg.

In further elucidation of the curve I may say that the critical (steep) part was covered by several determinations for each point. Thus there are five determinations averaged for the positions between 13 and 15. Seven between 15 and 18, five between 18 and 20, and six between 20 and 21. The determinations beyond 21 are single determinations. For the first part of the curve up to 13, there are numerous determinations. There are great variations in the single determinations compared with one another; these averages must therefore be regarded only as approximate values. With a sufficiently large number of determinations the irregularities between 15 and 17 and between 19 and 22 would no doubt disappear. But it is improbable that the general form of the curve would undergo any essential change even with a much more extensive series of determinations.

3. *Curves of Successive Half-dilutions.*

In contrast to these results, and for the purpose of defining the character of the main problem sharply, we may next consider the fertilizing power of a series of half dilutions of a 1 per cent. sperm suspension. The curves from these experiments furnish an almost incredible contrast to the one already given; as an example we may examine the following strikingly regular curve, Fig. 2. The first member of this series was a 1/8 per cent. sperm suspension freshly prepared, thus falling in position 3 on the scale; 8 c.c. of this was taken (No. 1); to 4 c.c. of 1, 4 c.c. of sea-water was added (No. 2) = 1/16 per cent., $(1/2^4)$; to 4 c.c. of 2, 4 c.c. of sea-water was added (No. 3) = 1/32 per cent., $(1/2^5)$; this was continued eight places to $1/2^{10}$. Four drops of a 10 per cent. egg-suspension was then added to each, and the percentage of segmented eggs was counted three hours later. Plotted they give the above curve. In this case it will be seen that the fertilizing power almost ceases at $1/2^{10} = 1/1024$ per cent. sperm suspension. The eggs and sperm were not at fault



CURVE 2.

because a parallel control series, in which the same quantities of the same lot of eggs were first placed in the same quantities of sea-water and sufficient of the original 1 per cent. sperm suspension added to make similar sperm dilutions, showed over 95 per cent. cleavage in each case, and actually 99 per cent. in No. 8 of the control where the sperm dilution was 1/1200 per cent. As a further control it may be added that eggs which fail to fertilize in such relatively concentrated sperm suspensions may all be fertilized by the subsequent addition of a trace of perfectly fresh sperm.

The type of experiment just cited was the first undertaken, and for a time it seemed to offer an almost insoluble problem, though the real explanation turned out to be extremely simple. I have twenty curves from similar experiments, fourteen of which run out absolutely from the third to the twelfth place on the scale (*i. e.*, from 1/8 per cent. to 1/4096 per cent.); in the remaining 6 (as in the curve just given) the dilutions were not carried far enough to reach the zero point, but they agree in principle with the others.

A number of control experiments demonstrated the relative lack of significance of the actual sperm concentrations. As one of these I may mention experiment C of August 3. In this case a series of sperm dilutions in powers of 4 was made from 1 per cent. The proportion of eggs fertilized ran off to 1 per cent. at $1/4^5$ $(1/2^{10})$ and to 0 at $1/4^6 = 1/4096$ per cent. But one drop of a 0.1 per cent. suspension of the original 1 per cent. sperm added to eggs in 200 c.c. sea-water fertilized 94 per cent. of them (control for sperm). Thus the control fertilized almost perfectly at 1/60,000 per cent. dilution, whereas the fifth member of the series of dilutions $1/2^{10}$ (1/1024 per cent. sperm) fertilized only 1 per cent. The actual concentration of the sperm is thus not the most significant thing.

This is also brought out strikingly in the following experiment (August 14). A series of half sperm dilutions was made as usual (Series *A*) 2 c.c. in each dish; to a second series (series *B*) of dishes was added 2 c.c. sea-water each and 4 drops of an egg-suspension. The numbers of series *B* were then inseminated by one drop each of sperm from the corresponding number of *A*, thus diluting the sperm about 1/60. To each of the *A* series (except 1) four drops of the same egg-suspension was then added. The resulting percentages of fertilization are given in Table I.

Table I.

	A.			*B.*	
1.	(1%)	–	1.	(1/60%)	– 99%
2.	(1/2%)	– 96.5%	2.	(1/120%)	– 99.5%
3.	(1/4%)	– 99.5%	3.	(1/240%)	– 98%
4.	(1/8%)	– 99%	4.	(1/480%)	– 60.5%
5.	(1/16%)	– 98.5%	5.	(1/960%)	– 51%
6.	(1/32%)	– 96.5%	6.	(1/1920%)	– 8.5%
7.	(1/64%)	– 21%	7.	(1/3840%)	– 1.5%
8.	(1/128%)	– 6%	8.	(1/7680%)	– 0
9.	(1/256%)	– 3.5%	9.	(1/15360%)	– 0

If we compare *A* and *B* in this table it will be seen that while it is true that *B* runs out earlier than *A*, nevertheless the fertilizations in the two series are not proportional to concentrations of sperm; for instance *A* 9 at 1/256 per cent. fertilizes 3.5 per cent. of the eggs, whereas *B* 3 at 1/240 per cent. fertilizes 98 per cent., *B* 4 at 1/480 per cent. fertilized 60.5 per cent., *B* 5 at 1/960 per cent. fertilizes 51 per cent. It is obvious that it is not concentration but *condition* of the sperm that is significant, which comes out with extreme emphasis in a control of this series. In this control, 1 drop of *A* (1 per cent. sperm) was added to 8 drops of the same egg suspension in 1,000 c.c. of sea-water 2 minutes after the other inseminations, thus making a 1/30,000 per cent. (1 × 1/1000 × 1/30) sperm suspension; every egg fertilized; the percentage of cleavage was 100 per cent.

The question then arises, what is this condition of the sperm which causes such loss of fertilizing power? We may note the following points: (1) To bring out the lack of significance of the absolute concentration of the sperm, in several of the experiments with successive half dilutions, counts were made of the numbers of spermatozoa seen in the egg-jelly of members of the series with no fertilizations: Thus on July 16 a series of half dilutions ran out to 0 in the seventh crystal (1/128 per cent. sperm): in ten eggs selected at random from this crystal, an average of 9 spermatozoa was counted in the jelly and in contact with the membrane of these eggs; but, as the upper and lower surfaces could not be examined, the whole number must have been at least double; in No. 8 of the series, an average of five spermatozoa was counted with each egg; in No. 9 an average of 1.2; No. 10, 1.4; No. 11, 0.9. Similar counts were made in other cases. But in fertilizations under optimum conditions all of the eggs may fertilize in dilutions of sperm so great that it is almost impossible to find spermatozoa in the jelly of the eggs. (2) The spermatozoa are active and the eggs readily fertilizable in such a series as the above. Repeated observations were made on this point; which would be tedious to relate in detail.

It may be noted that in the fertilization under optimum conditions the eggs were first placed in sea-water, and given quantities of sperm then added; whereas in the experiments with successive dilutions eggs were added to sperm suspensions already made up. This suggested that the order of adding eggs and sperm might be of significance in some way. However, this does not appear to be the case.

The possibility remained that the repeated handling of the sperm in successive dilutions decreased their motility. Microscopical examination did not confirm this idea; and subsequent experiments disproved it, as the fundamental factor at least.

Thus it would appear that the only real difference between the optimum and minimum conditions of the fertilizing power of sperm dilutions is a time factor; under what I have called the optimum conditions the final dilution is made from a relatively concentrated sperm suspension in the presence of eggs; but under the conditions of successive dilutions time elaspses before the eggs are added.

Thus in Curve 2 the preparation of the series of sperm dilutions from the original 1 per cent. suspension occupied 22 minutes before the eggs were added. In Curve 1, on the other hand, less than a minute elasped from the time of preparing the 1/100 per cent. and 1/1000 per cent. sperm suspensions used in the last four determinations to the time of their use in inseminating (see p. 234); and the final dilution was made in the presence of the eggs.

The time factor is the real explanation as will be shown immediately. But at first sight it did not seem a very probable explanation for two reasons: in the first place the time from preparation of the original 1 per cent. sperm suspension to that of addition of eggs is usually less than twenty-five minutes, which is usually considered too short a time for injury to sperm; and in the second place, after the addition of eggs to the sperm-dilution series, in several control experiments the original 1 per cent. sperm suspension was shown to be capable of fertilizing at 1/30,000 of 1 per cent. ($1/2^{15}$ ca.) by addition to eggs in sea-water. *If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration.* As soon as such a proposition is formulated it is easily tested experimentally, and this was done in a thorough fashion.

4. *Time as a Factor in the Fertilizing Power of Sperm Suspensions of Different Concentrations.*

The experiments under this head were performed in three ways: *A*. A considerable quantity of the sperm suspension to be tested was made up, and divided in several equal parts in a series of bowls; measured equal quantites of the same egg-suspension were then added to members of the series at definite time intervals. This method was followed for sperm dilutions from 1/300 per cent. (between 8 and 9 in the scale) down. *B*. Measured amounts of the more concentrated sperm suspensions were added at time intervals to measured quantities of eggs in equal amounts of sea-water. *C*. Finally, to control the data in section 3, a series of sperm suspensions, made by successive half dilutions as in section 3, was divided in two equal series, and eggs were added at once to the one series, and after a time interval to the second.

A. The following table gives the data under method *A*. The figures at the head of each vertical column give the sperm dilution in fractions of the 1 per cent. sperm suspension; below is given the place of such a sperm suspension in the scale of powers of 2. The figures in the columns give the percentages of fertilizations for inseminations made at the time (age of the suspensions in minutes) indicated at the left. To illustrate the method of experimentation for one column which will serve for all the rest,

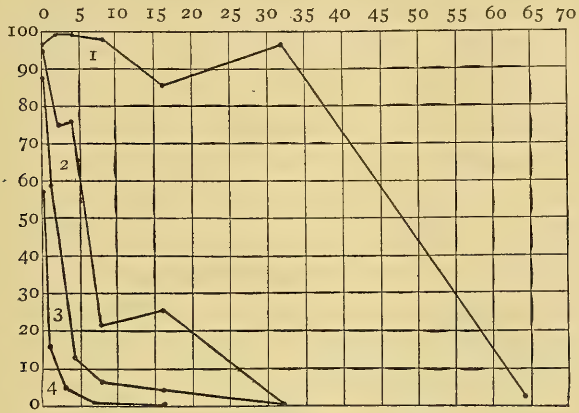
TABLE II.

SHOWING RATE OF LOSS OF FERTILIZING POWER OF SPERM SUSPENSIONS.

	$\frac{1}{300}$	$\frac{1}{600}$	$\frac{1}{1200}$	$\frac{1}{2400}$	$\frac{1}{3000}$	$\frac{1}{3600}$	$\frac{1}{6000}$	$\frac{1}{12000}$	$\frac{1}{18000}$	$\frac{1}{24000}$	$\frac{1}{30000}$	$\frac{1}{60000}$	$\frac{1}{120000}$	$\frac{1}{240000}$
0	96	100	99.5	95	95.5	97	91	97	....	97.5	88	91.5	58	40
1	....	....	99.5	49.5	.....	52	.....	....	....	.....	....	42	16	6
2	99.5	98.5	.....	....	75	....	80	92.5	98	99.5	59.5	....	.....	....
3	....	....	100	91.5	.....	88.5	.....	.....	....	.....	....	....	4	16
4	99.5	95.5	.....	....	76	....	34.5	93.66	85.5	49.5	13	15.5	.....	....
6	....	....	93.5	52	.....	80.5	.....	.....	....	.....	....	....	0.5	1
8	98.5	65.5	.....	....	21	....	28.5	54.5	73.5	67	7	17	.....	....
16	85.5	?	99.5	45	26	16.5	.....	.....	14	36	4.5	1	0	0
32	96.5	67.5	30.5	1.5	0	4.5	9	1	0	4	0	0.5	0	0
64	2.5	19	0	0.5	0.5	3.5	2	0	0	4	0	0	0	0
120	8.5	3	.....	....	.....	....	.....	.....	....	.....	....	....	.....	....
	8–9	9–10	10+	11+	11–12	12−	12–13	13–14	14+	14–15	15+	16−	16–17	18−

we shall give the experimental data for 1/30,000 per cent.: August 18, 1914. The eggs of two females were taken at 9.50 A.M. and washed at 10.04, 10.06 and 10.23 (150 c.c. of sea-water being used in each washing). A series of 7 Syracuse crystals was then laid out with 10 c.c. of sea-water in each. To 1 was then added 5 drops of the egg-suspension. A single drop of fresh dry sperm was then added to 333.3 c.c. sea-water at 10.37 making a 1/100 per cent. sperm suspension, and 1 drop of this was added to crystal 1 at 10.37.30 and stirred in by an assistant making a 1/30,000 per cent. (1/100 × 1/10 × 1/30) sperm suspension in presence of eggs. One drop of the 1/100 per cent. sperm was also added to crystals 2–7, which contained no eggs, at 10.38, making 1/30,000 per cent. sperm suspension in each. To No. 2, 5 drops of the same egg-suspension was added at 10.40, to No. 3 at 10.42, to No. 4 at 10.46, to No. 5 at 10.54, to No. 6 at 11.10, to No. 7 at 11.42. At 2.30 P.M. my assistant, Mr. Cohn, then estimated the percentages of segmented eggs in each crystal, by first thoroughly mixing the eggs, then assembling them, taking a sample, and making two counts of 100 each, which were averaged.

The table shows (1) that the effect of time up to 64 minutes is to diminish the fertilizing power of the suspensions at every dilution represented. (2) That the rate of loss of fertilizing power increases with dilution, *i. e.*, the effect of time varies inversely to concentration of sperm. This is brought out very clearly by the following curves (Fig. 3) of loss of fertilizing



CURVE 3.

power of sperm suspensions at different concentrations. The abscissæ represent age of sperm suspensions in minutes; the ordinates represent fertilizing power as expressed in percentages of segmenting eggs. Each curve stands for a given sperm dilution. Curve 1 represents loss of fertilizing power of a 1/300 per cent. sperm suspension, curve 2 of a 1/3000 per cent., curve 3 of a 1/30,000 per cent. and curve 4 of a 1/120,000 per cent. sperm suspension.

B. On August 6, I prepared a series of seven sperm dilutions in powers of 4 from 1 per cent. to 1/4096 per cent. Each of these was then used to fertilize a measured quantity of egg-suspension at the intervals given in Table III.

For the fertilizations 10 c.c. sea-water was measured out in advance in Syracuse crystals and 5 drops of a 5 per cent. egg suspension added to each. For each fertilization 1 drop of sperm was added and stirred in. It will be observed that 1 per cent. sperm lost none of its fertilizing power so far as this test went; 1/4 per cent. fell off from 96.5 per cent. to 16.3 per cent.; 1/16 per cent. from 46.5 per cent. to 0; 1/64 per cent. from 0.5 per cent. to 0 in the second place; whereas the greatest dilutions did

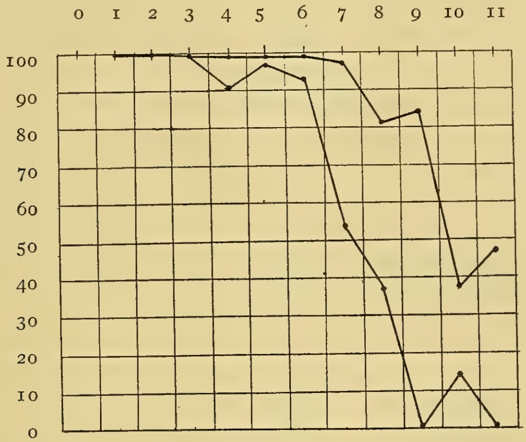
TABLE III.

Sperm Dilution.	Made at	Fertilizations.				
		A.	*B.*	*C.*	*D.*	*E.*
		2.50 P.M.	3.01 P.M.	3.16 P.M.	3.35 P.M.	4.03 P.M.
1. 1%......	2.20 P.M.	99 %	100%	100 %	99.5%	99.5%
2. 1/4%.....	2.28 P.M.	96.5%	68%	61.5%	18.5%	16.3%
3. 1/16%....	2.30 P.M.	46.5%	18%	8.5%	4 %	
4. 1/64%....	2.32 P.M.	0.5%	0%	0%	0 %	
5. 1/256%...	2.34 P.M.	0	0%	0%		
6. 1/1024%..	2.36 P.M.	0	0%			
7. 1/4096%..	2.39 P.M.					

not fertilize at all. It should of course be noted that the sperm suspensions used were diluted 300 times in the actual insemination (10 c.c. = 300 drops of sea-water, and one drop sperm added).

Thus time is an important factor in the fertilizing power of sperm dilutions from 1/4 per cent. down. The matter cannot be stated with great accuracy, but we can say in general (referring to Table II.) that sperm suspensions from 1/300 per cent. to 1/1200 per cent. lose their fertilizing power nearly completely in 64 minutes; from 1/2400 to 1/24,000 in 32 minutes; from 1/30,000 to 1/60,000 in 16 minutes; from 1/120,000 to 1/240,000 in 6 minutes. Table III. shows loss of fertilizing power of higher concentrations by a different method.

C. We are now in a position to understand the principal reason why the curves of successive half or quarter dilutions of a 1 per cent. sperm suspension run off so rapidly. The reason is that the preparation of the series requires time, 10 to 20 or more minutes. In the early experiments of this kind the significance of brief periods of time was not recognized, and so no time records were kept; but I have 13 curves with accurate time records. Of these I reproduce only two (Fig. 4). For this experiment (Sept. 5), (1) 6.6 c.c. of 1 per cent. sperm was prepared, 9.30 A.M. (2) 4 c.c. of 1 was transferred to a Syracuse watch crystal and 4 c.c. of sea-water added (= ½ per cent.). (3) 4 c.c. of 2 was transferred to crystal 3 and 4 c.c. sea-water added (= ¼ per cent.) and this was continued to 12 numbers (finished at 9.39.30 A.M.). The suspensions 2–12 was then divided in two equal amounts of 2 c.c. each, making series *A* and *B*. To each crystal of series *A* 2 drops of a 1 per cent. egg-suspension were added at 9.47, that is, 17 minutes after the 1 per cent. sperm was prepared. Twenty-eight minutes later (10.15 A.M.) two drops of the same egg suspension were added to series *B*. The only difference between series *A* and *B* is the time factor. The percentages of cleavages were counted for both series, and the plotted results given the curves. Considerable loss of fertilizing power has occurred in series *B* as compared with series *A*. Now, if we compare these time intervals with those given in Table II. we see that, in the curve of series *B* the last number, which is a $1/2^{11}$ or 1/2048 per cent. sperm suspension, loses its fertilizing power completely in 36 minutes (*i. e.*, from 9.39.30 to 10.15.30) which corresponds very well with the rate of loss of fertilizing power in a 1/2400 per cent. sperm suspension. This agreement is rather closer than usual; in some cases the series of ½ dilutions ran out at higher concentrations in about the same time; but in no case, I think, did they require more time. This suggests some possible stimulating effect of the successive changes which causes the spermatozoa to lose their fertilizing power more rapidly than under the time factor alone.



Curve 4.

Gemmill (1900) observed that the duration of vitality of spermatozoa of sea-urchins and limpets tested by their movements or by the fertilizing capacity, varies greatly "according to the amount of sperm used in proportion to the volume of sea-water in which it was shed." "When a small quantity of sperm was mixed with a large quantity of sea-water, the duration of vitality of the spermatozoa is short, but when the converse proportions are used, it is greatly lengthened." "By taking sperm from a sea-urchin and mixing it in different vessels with different quantities of sea-water, one obtains sets of spermatozoa, which will retain their vitality for a rising series of terms, *e. g.*, 8, 12, 16, 24, 48 and 72 hours. For the longest term, the proportion of spermatic fluid to sea-water should be not less than 1 to 10."

Gemmill was thus dealing with the same phenomenon with which we are concerned. He gives, however, no exact quantitative data and relatively few experiments were performed. He attributes the results to (1) greater activity of the spermatozoa, and consequent earlier exhaustion in the more dilute suspensions and (2) to dilution of the "spermatic fluid" by which he supposes the spermatozoa to be nourished.

5. *Other Factors in the Fertilizing Power of Sperm Suspensions.*

In the large number of experiments carried out to test the fertilizing power of sperm suspensions the general form of the curves is remarkably constant. Some, however, are quite irregular, and it was never possible to get *exactly* the same curve in the repetition of any experiment. A few of the irregularities may conceivably be due to error, as for instance the accidental presence of some toxic substance in one of the dishes of a series, though painstaking care was used to avoid such sources of error. The failure to obtain exactly the same curve in different experiments is no doubt also due in part to the natural variability of different lots of eggs and sperm.

In an attempt to discover the sources of variation and error, the effect of egg concentration, *i. e.*, the absolute quantity of eggs in a given bulk of a sperm suspension of given strength was tested. On the whole the effect of egg-concentration was found to be relatively small within so wide a range that it cannot be regarded as a large factor in the variability of the curves; because the egg-concentration of the curves was always below the point where it was demonstrably a limiting factor. Tests were made of sperm suspensions ranging from 1/62.5 per cent. to 1/8,000 per cent. But it was only from about 1/500 per cent. down that any considerable effect was observed within the range of egg concentration employed.

The method of the experiments tabulated (Table IV.) may be given for 1/500 per cent. sperm as it was the same for the others August 31: A quantity of 1/500 per cent. sperm suspension was freshly prepared 10.54.30 A.M., 2 c.c. of this was then placed in each of seven crystals (1–7). From 10.56.30 to 10.59 A.M. eggs were added as follows: to 1, one drop of a 1.75 per cent. egg-suspension, to 2 two drops, to 3 four drops, to 4 eight drops, to 5 sixteen drops, to 6 one c.c., to 7 two c.c. The numbers in the table give the percentages of segmented eggs. The tests with 1/1,000 per cent. and 1/2,000 per cent. sperm were made with the same egg suspension. For the tests with 1/4,000 per cent. and 1/8,000 per cent. sperm a 3.3 per cent. egg-suspension was used. Thus for each series the egg-concentration is approximately doubled in successive numbers of the series (in No. 7 = 64 times No. 1).

TABLE IV.

EFFECT OF EGG-CONCENTRATION ON THE FERTILIZING POWER OF SPERM SUSPENSIONS.

Sperm Suspensions.	Egg-suspensions.						
	1.	2.	3.	4.	5.	6.	7.
1/500%	100	99	99.5	97	93.5	82.5	56
1/1000%	97.5	94.5	93	76.5	48?	76.5	33.5
1/2000%	96.5	83.5	75	72.5	42.5	36	32
1/4000%	79.5	66.5	42.5	47.5	12.5	16	4.5
1/8000%	46.5	52	80?	30.66	27	15.5	7.5

The percentages of fertilization fall off in each of these sperm suspensions with increase of egg-concentration, and the amount of falling off increases in general with the dilution of the sperm. There was certainly no numerical deficiency of spermatozoa in the highest egg-concentrations; the reason for the falling off therefore appears rather obscure, and as it is not involved in the present problem, I shall not discuss it here. But as the egg-concentration employed in any of the preceding experiments did not exceed that of column 3, and the same egg-concentration was always employed throughout any experiment, it is obvious that the effect to be attributed to the egg-concentration employed in the preceding experiments is very small.

III. Discussion.

Within a wide limit of egg-concentration the important factors in fertilizing power of sperm suspensions are: (1) concentration, (2) time. A third factor, which is not of equal significance to the other two, is the given variability of the reproductive elements. Such variability attaches of course both to ova and spermatozoa; in general it will affect only absolute values for given combinations, and not at all the relative values found in any single experiment. Moreover, as it is a chance factor, it will tend to be eliminated in a series of determinations. Fortunately both eggs and spermatozoa of *Arbacia* are relatively very constant materials if care be taken to wash the eggs thoroughly, and if the factors of concentration and time are fairly constant for the sperm. For the eggs these two latter factors are of such slight importance within the given limits as to be practically negligible. The significance of the concentration factor for the fertilizing power of sperm is of course obvious without discussion. We therefore turn to the time factor.

The most significant aspects of the time factor are, first, the unexpectedly rapid rate of loss of fertilizing power of sperm suspensions, and second the increase of rate of loss with dilution. There are but two ways of explaining these facts: either (1) the motility of the spermatozoa is quickly reduced in sperm suspensions to such an extent that they cannot bore into the egg or (2) the spermatozoa lose some substance essential for the fertilization reaction.

The following are the objections to the first alternative: (*a*) Microscopical examination lends it no support; I have repeatedly observed, that fertilizing power of sperm cannot be expressed either in terms of motility, or of success in penetrating the jelly of the egg and coming in contact with the membrane. In the experiments on successive half dilutions (p. 238) I kept records, in several series, of the numbers of spermatozoa in the jelly of unfertilized eggs, and found in some cases an average of 9 spermatozoa visible in the jelly, or on the membrane of certain lots of eggs none of which had fertilized; this could not be more than half of the spermatozoa in association with such eggs; and other observations made immediately after insemination demonstrated the high degree of motility of spermatozoa of entirely barren sperm suspensions.[1]

These observations contrast in the most striking manner with the fact that not a single spermatozoön can be seen in the jelly of eggs fertilized with highly dilute fresh sperm suspensions, where, nevertheless, nearly every egg may be fertilized.

(*b*) Penetration of the egg is not solely a function of motility of the spermatozoön. Penetration follows, as a matter of fact, after the fertilization reaction has begun, and it is due to the inception of such reaction, not the reverse as is commonly assumed.[2] In *Nereis*, as I have previously described, penetration does not take place until 45 to 50 minutes after insemination and the initiation of the fertilization reaction. The facts described in this paper show that in *Arbacia* no penetration takes place unless the sperm has started the fertilization reaction; if this does not take place, the spermatozoön remains external, however active it may be. And if it does occur the initiating spermatozoön is speedily engulfed by the egg.

(*c*) It is not easily understood on this theory why dilute sperm suspensions should lose their fertilizing power more rapidly than more concentrated suspensions;[1] the relative freedom from CO_2 and other sperm excreta should favor a longer continuation of their motility in the dilute suspensions rather than the reverse.

---

[1] Glaser's experiments (1915) would bear the interpretation that, in those cases of normal insemination described by him in which fertilization does not occur except in the presence of several spermatozoa for each egg, the time factor which I have just described was operative. In other words that the majority of the spermatozoa in question had lost their receptors. But in the absence of exact data as to age and concentration of the sperm suspension, it cannot be asserted that this is the correct interpretation although I obtain exactly the same results in my time series (p. 238). My dilution experiments prove beyond a doubt that a single spermatozoon suffices for the whole process of fertilization under optimum conditions (defined on p. 233). Glaser's experiments, however, raise the question whether the efficacy of heavy insemination in the case of a stale sperm suspension is due to mass action, or to the survival of a small percentage of effective spermatozoa? So far as I can see this question can not be answered on the basis of our present information.

[2] Spermatozoa may penetrate into unripe ovocytes in some cases, as has been noted by several observers; in such a case there is no fertilization reaction. In the present experiments the unfertilized eggs were not penetrated by the spermatozoa.

(*d*) Moreover, in general the results of recent fertilization studies such as the antagonistic action of sperm suspensions of different phyla, inhibition of fertilization in the presence of blood of the species, or in the absence of certain ions (Loeb, '14), or again the sterility in certain self-fertilizations, and finally the inability of spermatozoa to penetrate fertilized eggs, unite in demonstrating the relative lack of significance of motility as such.

We come therefore to the conclusion that the individual spermatozoa in suspension tend to lose their fertilizing material, so that an increasing proportion of these spermatozoa become absolutely ineffective whatever their motility. This conclusion is in agreement with all the data of the foregoing experiments, and seems to be the only one competent to explain the results.

The following questions arise: (1) Whether the loss of this substance by the sperm is a mere process of diffusion or an active secretion? (2) Can the substance be recovered from the fluid of the suspension, or can its presence in the fluid be demonstrated in any way?

As regards the first question: In the case of the ova we know that the external jelly-covering is loaded with sperm-agglutinating substance which diffuses into the sea-water continuously. It is theoretically possible, at least, to apply a similar conception to the spermatozoön, although no such covering is demonstrable. The more rapid loss of fertilizing power in the greater dilutions would be consistent with this interpretation. From this point of view we would have to regard the sperm head as covered superficially with a layer of fertilizing material, like the phosphorus on a match. Such a conception is by no means impossible. On the other hand the fact that dilutions reached by a series of successive half-dilutions from 1 per cent. lose their fertilizing power more rapidly than the same dilutions made in one stroke, indicates that successive stimulation hastens the loss, which therefore appears more in the nature of a secretion or a discharge than mere diffusion. The source of the substance must ultimately be the sperm cell itself, and it is quite possible that, as in the case of the egg, there is both a superficial layer and an internal supply.

---

[1] Gemmill (1900) observed the same phenomenon and concluded that the more rapid exhaustion of spermatozoa in dilute suspensions is due to dilution of a hypothetical nutritive medium which keeps the spermatozoa of concentrated suspensions in a vigorous condition. This explanation comes back to the principle of loss of motility, so far as it relates to fertilizing power.

It must be admitted that the data are inadequate to answer this problem. The statement of the problem can therefore serve only to bring out the resemblance between the spermatozoön and the ovum in respect to the existence of a fertilizing substance in each, the fertilizin in the case of the ovum and the sperm receptors in the case of the spermatozoön, and also the possible resemblance in respect to the disposition of the substances in each. It certainly is an interesting parallelism that both cells contain a substance necessary to fertilization, which may be lost in the sea-water.

The most interesting and crucial question of course concerns the possibility of detecting this lost substance in the fluids of the suspensions. If such a substance actually occurs in the fluid it should have the property of fertilizing ova; unless it can be detected by this property, we have no other indicator for it. So far I have not been able to make even a beginning on this problem. As is well known a number of experimenters have attempted without success to derive a fertilizing medium from spermatozoa. It has been suggested by Loeb that the reason for the failure to secure an extract of spermatozoa that will fertilize is that the motile power of the spermatozoön is needed to carry the effective substance into the egg. But it may equally well be that the methods hitherto employed have been too brutal; the substance may well be too labile to withstand extraction by ether, etc.

My results strongly suggest, if they do not prove, that such a substance must be present in the fluid of sperm suspensions of *Arbacia*, and they therefore suggest other methods for securing it for testing. We must bear in mind that it can form only an extremely small proportion of the entire spermatozoön, as proved by morphological considerations alone, and that it must be superficial in position and easily detached as proved by its effectiveness before the spermatozoön penetrates. Extracts of the entire spermatozoön must contain numerous other substances which may neutralize its effectiveness.

The difficulty of the investigation as shown by my experiments is that it is liberated only very slowly in concentrated suspensions and that its amount in dilute suspensions would presumably be too slight to be effective. Some means can probably be devised for liberating it in concentrated sperm suspensions and freeing it of the spermatozoa for testing.

Finally I may point out that the conclusion that spermatozoa lose a substance necessary for the exercise of their fertilizing power is consistent with my own point of view of the mechanism of fertilization as well as with Loeb's. From my point of view the spermatozoön loses its receptors, viz., the substance that activates the fertilizin of the egg; from Loeb's point of view the spermatozoön loses its lysin, the substance that corrodes (cytolyzes) the egg.

My previous experiments had shown that eggs produce a certain substance in sea-water (fertilizin) which is necessary for their fertilization; fertilized eggs no longer produce this substance and are incapable of fertilization. Both eggs and spermatozoa therefore contain substances, more or less liable to loss, which are necessary for fertilization. The mechanism of fertilization cannot possibly, therefore, be regarded in the simple manner postulated by Loeb's theory. The existence of parthenogenesis demonstrates the efficacy under given conditions of the egg-substance alone; we must therefore regard the spermatic substance essentially as an activator of the fertilizin of the egg.

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BIOLOGICAL BULLETIN

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ON THE RHYTHMICAL SUSCEPTIBILITY OF DEVELOPING SEA URCHIN EGGS TO HYPERTONIC SEA WATER.

ARTHUR RUSSELL MOORE.

(From the Biological Laboratory of Bryn Mawr College and the Marine Biological Laboratory at Woods Hole.)

In a recent communication M. Herlant[1] attempting an analysis of Loeb's method of artificial parthenogenesis concludes that (1) the fatty acid treatment gives rise to the rhythmical activity of the centrosome but never to normal divisions of the egg; (2) the treatment of the eggs with hypertonic sea water causes the formation of accessory asters and is necessary to complete the causes for normal division; (3) the optimum results are obtained by applying the hypertonic solutions at certain intervals after fatty acid treatment, viz., 30 and 70 minutes and possibly 115–120 minutes, while with 40–50 and 95–100 minute intervals marked minima are shown.

As to the first generalization, Herlant ignores the fact that in *Strongylocentrotus* and *Arbacia* the fatty acid treatment alone may cause normal segmentation. If the eggs of *S. purpuratus* are kept at a low temperature (5°–10°) after acid treatment alone, they divide regularly and may reach the morula stage.[2] In *Arbacia* eggs, normal segmentation may take place after acid treatment without subsequent treatment with the hypertonic solution, but does not as a rule proceed beyond the two-cell stage. On the other hand hypertonic treatment alone may cause *Arbacia* eggs to segment and develop into swimming larvæ, while it brings about only early segmentation stages in the eggs of *Strongylocentrotus*.

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[1] M. Herlant, *Comptes Rendus de l'Academie*, T. 158, p. 1531.

[2] J. Loeb, "Artificial Parthenogenesis and Fertilization," p. 76.

Herlant apparently assumes that the action of the hypertonic solution must be subsequent to the acid treatment, that to be effective such action must occur in a certain phase of the rhythmical activity of the centrosome. Since in *Strongylocentrotus purpuratus* treatment of the eggs with the hypertonic solution may precede that with acid sea water by as much as a forty-eight hour interval with the result that normal parthenogenetic larvæ are formed,[1] Herlant's contention is not justified. Furthermore, treatment of the eggs with small quantities of KCN or depriving them of oxygen, may replace hypertonic treatment after fatty acid. Now lack of oxygen or the repression of oxidations does not cause aster formation, but on the contrary suppresses it. In fact it has even been shown that in *Arbacia* the first steps in development induced by the acid treatment may be reversed and the egg returned to its resting stage with its original possibilities of fertilization, simply by withholding oxygen from such an egg or by treating it with KCN.[2] In view of these facts, it seems evident that Herlant's conclusion that the hypertonic solution is a necessary factor in artificial parthenogenesis because it controls aster formation, does not hold.

As to the rhythmicity in effectiveness of the hypertonic treatment which Herlant found in his experiments, it seemed possible that the relation between the time spent in normal sea water after acid treatment and the time in the hypertonic solution might have a bearing upon the question. Six years ago the present writer found, in working with the eggs of *Strongylocentrotus purpuratus*, that if the exposure to normal sea water in such an experiment be lengthened, the subsequent treatment by hypertonic sea water must be shortened to secure optimum results.[3] Repetitions of the experiment at Woods Hole during the past summer, however, have indicated that the relation does not exist for *Arbacia*.

Table I. shows the results of dividing a lot of *Arbacia* eggs, after fatty acid treatment, into three parts which remained in normal sea water, 5, 25 and 90 minutes respectively, before being put into hypertonic sea water. In each case a portion of each lot was removed from the latter solution after 17½, 20, 25, 30, 35, and 40 minutes, and allowed to develop in normal sea water. The percentages given in the table show the degree of blastula development in each culture. Repetitions of the experiment showed no significant variation in the optimum exposure to the hypertonic solution with changes in the time the eggs remained in the normal sea water after acid treatment.

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[1] J. Loeb, *Journ. Exp. Zool.*, vol. 15, p. 201.

[2] J. Loeb, *Science*, N. S., Vol. 38, p. 749.

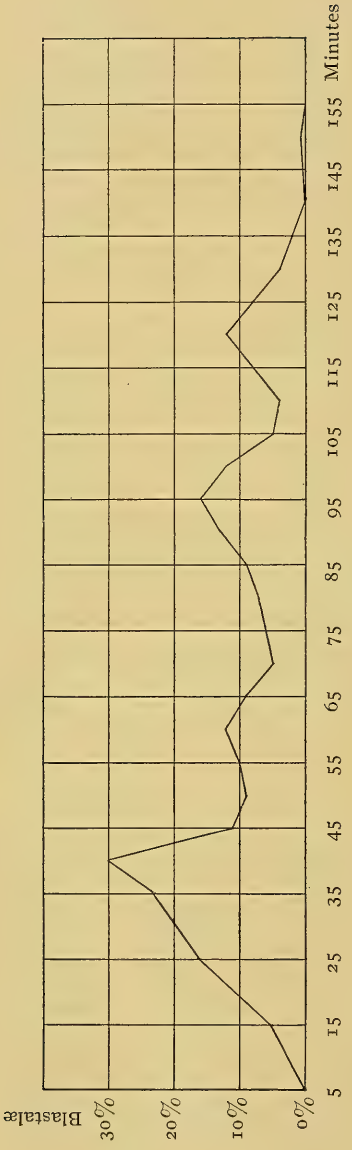
[3] J. Loeb, "Artificial Parthenogenesis and Fertilization," p. 96.

Table I.

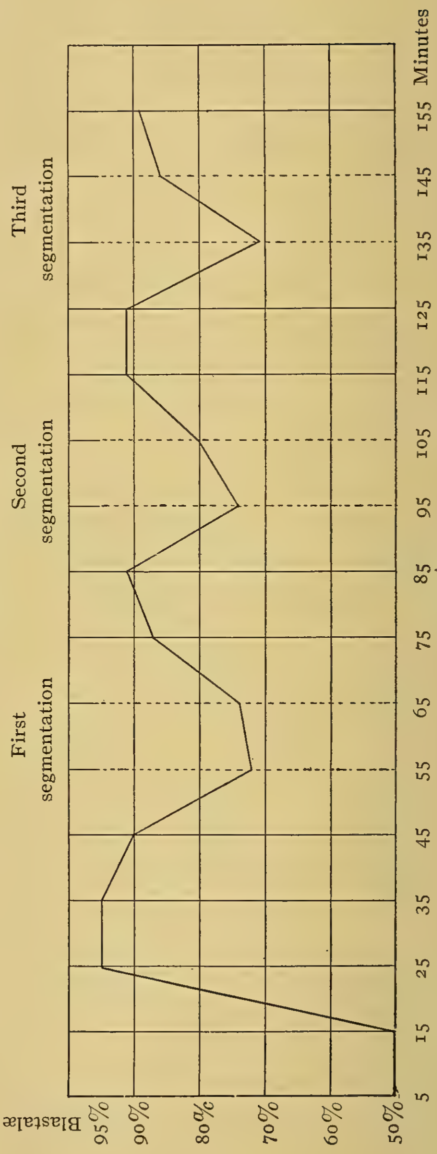
$T° = 20° - 22°$.

Eggs Remained in Normal Sea Water, After Butyric Acid Treatment	Percentages of Eggs Develop into Blastulæ After Exposure to Hypertonic Sea Water for					
	17½ Min.	20 Min.	25 Min.	30 Min.	35 Min.	40 Min.
5 minutes......	—	1%	5%	13%	24%	18%
25 "	—	8%	18%	24%	20%	8%
90 "	—	—	—	1%	3%	1%

In order to determine if the eggs of *Arbacia punctulata* which had been treated with acidulated sea water, showed a rhythmicity in sensitiveness to the hypertonic solution, such as Herlant's experiments with the eggs of *Paracentrotus lividus* indicated, the following experiments were carried out. The eggs of several sea urchins were collected, treated with sea water made acid by the addition of 2 c.cm. N/10 butyric acid to 50 c.cm. of sea water. After remaining in this solution for from 2 to 2½ minutes the eggs were transferred to normal sea water. At the end of 5-minute intervals lots were removed to finger bowls containing hypertonic sea water [50 c.cm. sea water + 8 c.cm. 2½ M ($NaCl + CaCl_2 + KCl$)]. After remaining in the hypertonic sea water for 25 minutes the eggs were put into normal sea water and allowed to develop. The percentages of advanced morulæ or non-swimming blastulæ were determined by counting random fields. The following table (Table II.) gives a typical result, showing optimum effects when the eggs were put into the hypertonic solution 40, 60, 90–100 and 115–125 minutes after acid treatment. The rhythmical character of the result is obvious from Curve I., where the ordinates indicate the percentage of larvæ formed, while the abscissæ indicate the time which elapsed between acid treatment of the eggs and their exposure to hypertonic sea water.



CURVE I.



CURVE II.

TABLE II.

$T^{\circ} = 19^{\circ} - 21^{\circ}$. Hypertonic exposure = 25 minutes.

Time in normal sea water after acid treatment	5	15	25	35	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110
Blastulæ per cent.	–	5	16	23	30	11	9	10	12	9	5	6	7	9	13	16	12	5	4

Time in normal sea water after acid treatment	115	120	125	130	140	150	165	175	185
Blastulæ per cent.	8	12	8	4	0	<1	0	1	0

It seemed possible that the normally fertilized eggs also might show a rhythmical susceptibility to hypertonic sea water. Especially did this seem probable in view of Lyon's experiments. He found that normally fertilized eggs of *Arbacia* gave alternating maxima and minima of susceptibility to heat, cold and lack of oxygen; and that CO_2 production was greatest at the time of cytoplasmic division.[1] E. G. Spaulding has shown a rhythmical susceptibility and immunity of fertilized *Arbacia* eggs to the effects of ether, HCl, KCl and NaCl. He found a rise in immunity up to the time segmentation begins, followed by a sharp decrease during cleavage, with a marked rise at the end of cleavage.[2] A. P. Mathews found an approximate rhythmicity in the behavior of *Asterias* eggs toward KCN solutions.[3]

In my own experiments the *Arbacia* eggs were fertilized in one finger bowl and at the end of each ten-minute interval a lot was removed to the hypertonic solution and kept there for 40 minutes, after which the eggs were returned to normal sea water to develop. The results of a number of such experiments indicate (Table III., Curve II.) that the maximal susceptibility occurs just after fertilization (5–15 minutes) and immediately before and during each cytoplasmic division, and that the maximal resistance is shown 35–45 minutes after fertilization and just after each division. This corresponds to Lyon's statement regarding the effects of heat upon dividing eggs, viz., that *Arbacia* eggs are especially sensitive to heat just before division and that they are most resistant after division.

---

[1] E. P. Lyon, *Am. Journ. Physiol.*, Vol. 7, p. 56, and Vol. 11, p. 52.

[2] E. G. Spaulding, BIOL. BULL., Vol. 6, p. 224.

[3] A. P. Mathews, BIOL. BULL., Vol. 11, p. 137.

Table III.

$T^\circ = 20^\circ$.

Time in normal sea water after fertilization	5	15	25	35	45	55	65	75	85	95	105	115	125	135	145	155
Blastulæ per cent	50	50	95	95	90	72	74	87	91	73	80	91	91	71	86	89

The character of the curve constructed from Table III. differs from that made from the data of Table II. It will be noted that in the latter case there is an early maximum and minimum, and a slight secondary maximum reached at about the time cleavage would take place if the eggs had been normally fertilized. The dissimilarity may be due to the fact that the hypertonic solution may have two effects on developing eggs, viz., beneficial and injurious. We have seen that the acid treatment alone leads to the early stages of development, and that if the temperature is kept low the eggs of *S. purpuratus* develop to the early blastula stage. But before the gastrula stage is reached, the embryos go to pieces. To all appearances the early morula stages are quite normal. Loeb has suggested that cleavage may be accompanied by the production of toxic substances which, accumulating with each successive division, cause the larvæ to sicken and die. The injurious effects of these substances may be prevented by treatment of the egg with a hypertonic solution, or the formation of the injurious substances may be inhibited by long hypertonic treatment before membrane formation. It has been shown that simply by preventing oxidations for a time instead of treating the eggs with hypertonic sea water, normal development may be secured. Hence, the hypertonic solution or lack of oxygen exercises a beneficial or curative effect on parthenogenetically developing eggs and allows the embryo to develop to maturity.

On the other hand there is an optimum time for the continuance of the action of the hypertonic solution, and if the exposure is continued longer the solution acts deleteriously and as a result development stops and the egg disintegrates. It becomes clear then that the hypertonic treatment may have one of the two opposite effects upon the egg, *i. e.*, beneficial or injurious, depending upon the duration of the treatment, or in other words upon the condition of the egg when treated.

In normal fertilization the sperm cell which fertilizes the egg accomplishes two things of immediate importance, viz., starts division and prevents the production of toxic substances during cleavage or inhibits their action. It is therefore impossible for the hypertonic sea water to exercise its protective action upon the normally fertilized egg. It can affect the egg only injuriously. Our experiments show that this injurious action of the hypertonic solution is most pronounced just preceding and during cytoplasmic division, and that such action is very slight immediately afterward.

In the case of artificial parthenogenesis the hypertonic treatment is much shorter than in the experiments just described, and hence we may consider its injurious effects excluded. The Curve I. representing the results of Table II. shows the rhythmicity of the beneficial effect of the hypertonic treatment, while the curve constructed from Table III. indicates only injurious effects. There is therefore no reason why the two curves should be identical in character, although each shows a rhythmicity of susceptibility to the action of hypertonic sea water.

According to Loeb the artificial membrane formation in artificial parthenogenesis starts the chemical phenomena which give rise to the process of cell division and development; but the process is incomplete or abnormal and leads to the disintegration of the egg unless a second treatment is added, usually a treatment with hypertonic sea water. Since, by the membrane formation, chemical or physico-chemical changes induced in the egg are rhythmical, it is intelligible that it should make a difference in which stage of the cycle the treatment with the hypertonic solution is supplied. This is presumably the explanation of Herlant's observation.

In conclusion I wish to express my best thanks to Dr. Frank R. Lillie for so generously giving to me the privileges of the Marine Biological Laboratory at Woods Hole, and to Dr. Jacques Loeb for much helpful advice and criticism.

ON THE CONDITIONS OF ACTIVATION OF UNFERTILIZED STARFISH EGGS UNDER THE INFLUENCE OF HIGH TEMPERATURES AND FATTY ACID SOLUTIONS.[1]

RALPH S. LILLIE.

INTRODUCTORY.

In a former paper[2] I showed that brief exposure of the unfertilized eggs of *Asterias forbesii* to temperatures of 32° to 38° resulted in membrane-formation, cleavage and development. With normal eggs and the proper times of exposure almost every egg developed to a free-swimming larval stage; this treatment thus forms a highly effective parthenogenetic method. The time of exposure to the warm sea-water required to produce these effects is definite (within a certain slight range of variation) for any given temperature and decreases rapidly as the temperature rises. Thus, as regards the least exposure necessary for the formation of typical fertilization-membranes: "At 33° exposure must be prolonged to two minutes; at 34° the minimum lies somewhere between 30 and 60 seconds, at 35° between 15 and 30 seconds, at 37.5° between 5 and 15 seconds, and at 40° momentary exposure (5 seconds) produces membranes in practically all eggs."[3] The exposure required to induce development to larval stages was found to be considerably longer than for simple membrane-formation; at 35° from 70 to 90 seconds was required, at 36° from 50 to 60 seconds, at 37° from 30 to 35 seconds, and at 38° about 20 seconds. The responsiveness of the eggs to this form of treatment was found to depend on the stage of maturation; warming before the dissolution of the germinal vesicle had begun was ineffective and in fact inhibited maturation entirely; the most favorable period lay between the breakdown of the germinal vesicle and the separation of the first polar body; after both polar bodies had separated development was imperfect and never proceeded far,—even membrane-formation then failed in many eggs.

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[1] From the Marine Biological Laboratory, Woods Hole, and the Biological Laboratory, Clark University.

[2] *Journal of Experimental Zoology*, 1908, Vol. 5, p. 375.

[3] *Loc. cit.*, p. 384.

Recent advances in the physiology of fertilization and artificial parthenogenesis have made it desirable to examine these effects of temperature in greater detail and to correlate them with the similar effects produced by other agents. During the past summer at Woods Hole I have accordingly re-investigated the changes in unfertilized starfish eggs following exposure for different periods to temperatures ranging from 28° to 36°, with especial reference to the differences in physiological effect resulting from differences in time of exposure to a given temperature (*e. g.*, 32°), and also with reference to the manner in which the time of exposure required to produce a given effect (*e. g.*, membrane-formation) varies at different temperatures. Determination of the temperature-coefficients of the processes underlying these effects is likely to afford indications of the nature of the fundamental changes concerned in the activation of the egg. Experiments on the effects of exposure to weak fatty acid solutions for different periods were also carried out; and on the action of high temperatures (32° to 34°) and fatty acid solutions on eggs which had previously been subjected to a membrane-forming treatment.

It is well known that the temperature-coefficients of a large number of physiological processes have been found similar to those of chemical reactions in general.[1] This result is to be regarded simply as an expression of the fact that the energy for such processes is usually chemical energy freed by oxidations or other reactions, whose rate accordingly determines that of the process in question. There are, however, many instances in which rise of temperature produces an entirely different kind of effect. Often a process exhibits a critical temperature below which it entirely fails to take place.[2] In such instances the process may show a very rapid acceleration through a range of a few degrees above the critical temperature; it is then clear that the change of temperature acts in some other way than simply by accelerating an already existing chemical reaction. This is the class of cases to which belongs the influence of higher temperatures in initiating development in starfish eggs. Such departures from the usual temperature-coefficients of physiological processes indicate the entrance of other factors, the nature of which may be partly inferred from the character of the temperature-coefficient. Thus, to take the case of the starfish egg: in order to induce development of all eggs to a larval stage by exposure to a temperature of 31° it is necessary to keep them at this temperature for a period of about 15 minutes; at 36° an exposure of only one minute is necessary. The physiological process, whatever its nature, which renders the egg capable of proceeding with its development, thus takes place about fifteen times as rapidly at 36° as at 31°. This high temperature-coefficient indicates that a physical rather than a purely chemical change—possibly a change of the same nature as that determining the liquefaction of a warmed gel—is responsible for the altered behavior of the egg. The time-relations show that some definite and progressive process, the end-effect of which is to remove the conditions hindering further development, is taking place in the egg during the entire 15 minutes at 31°. Exposure for the full period of fifteen minutes is necessary to bring this process to its completion, *i. e.*, to a stage at which the egg is in a position, when returned to sea-water, to continue automatically its development to a larval stage. If the exposure is only 5 minutes there is also a definite change in the egg; a typical fertilization-membrane is formed and there may be some irregular change of form or possibly a few abnormal cleavages, but the egg never develops far and soon dies. In this case the process of activation is evidently incomplete, and only a few of the early steps in development are carried out. If the exposure is too long (20 to 25 minutes) the egg also fails to develop; the process initiated by the higher temperature thus

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[1] For a summary account of researches in this field *cf.* C. D. Snyder: *American Journal of Physiology*, 1908, Vol. 22, p. 309.

[2] Examples of such processes are: inactivation of enzymes and toxines or destruction of microorganisms by heat; heat-coagulation of proteins, and dependent processes like injury or destruction of various cells by heat; onset of heat-rigor;

excitation of thermal sense-organs (*e.g.*, of frog's foot) and of certain vaso-motor and other temperature-regulatory mechanisms by heat; thermotactic responses.

gives rise to injurious conditions if it continues beyond a certain time. For each temperature, in fact (from 30° to 38°), there is a well-defined optimum duration of exposure which initiates favorable development in all normal eggs; also a briefer exposure which results in simple membrane-formation followed by breakdown; and a more prolonged exposure which renders the egg incapable of development. It is noteworthy that at each temperature the ratios of the durations required for these several effects are closely similar,—the optimum exposure being typically from two to three times that required for simple membrane-formation, and the maximum exposure (at which development to a larval stage just fails) about one and a half times the optimum.[1] This indicates that some single process, involving a critical change in the condition of the egg-protoplasm and having a characteristically high temperature-coefficient, underlies and conditions all of these effects. This process does not begin until a temperature of about 29° is reached, and proceeds slowly at that temperature, taking approximately 30 minutes to attain its completion. A rise of eight degrees accelerates it some hundred times. Such facts appear to narrow the range of possibilities very materially; they point clearly to some physical change,—of structure, colloidal aggregation-state, viscosity, etc. —rather than to one of a purely chemical kind, as constituting the critical process underlying the activation of the egg.

The experiments of the past summer have shown further that exposure to weak fatty acid solutions produces in the egg effects which are in all essential respects identical with those resulting from exposure to the above temperatures. Starfish eggs placed for one minute in sea-water containing *n*/260 butyric acid (2 c.c. *n*/10 butyric acid *plus* 50 c.c. sea-water) all form fertilization-membranes on return to normal sea-water; but if left without further treatment the eggs typically fail to cleave and soon break down without further development. Precisely the same effect is produced by brief exposure to warm sea-water, *e. g.*, three or four minutes at 32°. In either case it is necessary, in order to induce complete development of such eggs, to subject them to some second or supplementary treatment, such as exposure to hypertonic or cyanide-containing sea-water. The starfish egg can, however, be made to develop completely without the necessity of any such after-treatment, simply by sufficiently prolonging the exposure to the membrane-forming agent. An exposure of 8 minutes to 32° is followed not only by membrane-formation, but by cleavage and development of all normal eggs to larval stages (*cf.* page 271). Similarly, exposure to *n*/260 butyric acid for a sufficient period—varying from 6 to 10 minutes —also causes all eggs to cleave and develop to larvæ (*cf.* p. 282). Over-exposure, if slight, is followed in both cases by a decrease in the proportion of favorably developing eggs; and if well-marked, by complete failure of development and early breakdown. The only noteworthy difference that I have observed between the effects of the two agents is that the time-relations in the case of exposure to fatty acid have been somewhat more variable than in the case of exposure to a definite temperature such as 32°. Thus in some experiments eggs have exhibited a considerable proportion of favorable developments after only one minute's exposure to weak fatty acid solutions.[1] In such cases however the concentration of acid was somewhat higher (3 c.c. *n*/10 fatty acid *plus* 50 c.c. sea-water) than in the experiments described above. In last summer's experiments (in which the fatty acid was always used in *n*/260 concentration) the curves relating time of exposure to the proportion of eggs forming larvæ were virtually identical in form with the two agents,—a fact showing that the essential effects produced by both types of treatment are the same.

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[1] *Cf.* below, page 279.

The fact that a properly timed single exposure to warm sea-water or fatty acid solution causes complete development suggests that the necessity for a supplementary after-treatment (*e. g.*, with hypertonic sea-water), in the case of eggs in which fertilization-membranes have been formed by brief preliminary exposure to a cytolytic agent, depends simply on the incompleteness of the change induced in such eggs by the membrane-forming treatment. The fact that by sufficiently prolonging this treatment one can induce complete development in all eggs indicates clearly that the after-treatment produces in the egg effects which are physiologically of the same kind as those resulting from the membrane-forming treatment, and not qualitatively different as has usually been supposed. If this is so, we must conclude that hypertonic sea-water is favorable not because it exerts a "corrective" action different from that of the membrane-forming agent, but simply because it enables the process started by the first treatment and arrested at an unfinished stage to proceed to its completion. On this view the effects of the two successive treatments are simply additive. Apparently under the influence of the higher temperature or the fatty acid a certain definite process, which we may call the activation-process, is started in the egg. This process, if it proceeds to a certain definite stage, puts the egg in a condition to continue automatically its development to the formation of larvæ; but if the process is arrested too soon (by the return to sea-water), the egg is able to carry out only a few of the early steps of development, including membrane-formation and perhaps a few cleavages. The after-treatment merely causes the resumption of the process and carries it to its completion. The unitary character of the activation-process is further indicated by the fact that the temperature-coefficients for simple membrane-formation and for the complete initiation of development are the same, as will be shown below. If this conclusion is correct, it should be a matter of indifference whether the exposure for the required period to the high temperature or the fatty acid solution is continuous or discontinuous. It ought to be possible to form fertilization-membranes by brief exposure to warm sea-water or fatty acid followed by a return to normal sea-water, and then later to complete the activation-process by a second exposure to either agent for an appropriate time. This is in fact the case; all of the four combinations have been tried: brief treatment with warm sea-water followed by after-treatment for several minutes with either warm sea-water or butyric acid solution; and membrane-formation by butyric acid followed by warming or a second treatment with acid. All four methods give the same result, namely the development of a high proportion of eggs to larval stages. The effect of such second treatment is in fact indistinguishable from that of exposure to hypertonic sea-water or cyanide.

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[1] See the experiments described in my recent paper in the *Journal of Experimental Zoology*, 1913, Vol. 15, pp. 41, 42. Starfish eggs exposed for 1 minute to a mixture of 3 c.c. *n*/10 acetic or butyric acid plus 50 c.c. sea-water (*n*/176 acid) gave in several cases 20–30 per cent. of larvæ and in one case 70–80%.

The problem of the nature of the effect produced on the egg by hypertonic sea-water, or the other corrective agent employed to supplement the membrane forming treatment, thus appears in a simpler light. In the starfish egg, after membranes have been formed as above, an exposure to (*e. g.*) 32° or to weak butyric acid solution for several minutes constitutes a highly favorable form of after-treatment, producing the same effect on development as hypertonic sea-water or cyanide.[1] This makes it appear doubtful that two qualitatively distinct processes are concerned in the activation of other eggs like the sea-urchin egg, where some form of after-treatment, different from the membrane-forming treatment, has hitherto proved necessary in order to induce development in a high proportion of eggs. The conditions are unlikely to be fundamentally different in the two animals. In the starfish egg the "corrective" effect resulting from after-treatment by heat has the same high temperature-coefficient as the initial change underlying simple membrane-formation by heat.[2] This could hardly be the case if the two processes were qualitatively dissimilar; it indicates clearly that the same fundamental change in the egg-protoplasm furnishes the conditions for both the membrane-forming process and the "corrective" process. I have found that in the *Arbacia* egg temporary warming (1 to 6 minutes at 32°, 34° and 35°) does not cause development (except in very few cases) even if followed by hypertonic sea-water;[3] and there is no evidence that prolonged treatment with weak fatty acid solutions will cause complete development in this egg.[4] The only highly and invariably effective after-treatment hitherto discovered for the sea-urchin egg is hypertonic sea-water.[1] It would thus appear that the conditions in this egg differ considerably from those in the starfish; but the fact that a simple exposure to hypertonic sea-water, if sufficiently prolonged, has the same effect in inducing development as a briefer exposure to the same agent combined with membrane-formation by fatty acid, seems to indicate that the conditions are fundamentally similar in both types of egg, and that a unitary process underlies activation in both cases. The remarkable effectiveness of hypertonic sea-water with the sea-urchin egg would seem to be due to certain special largely incidental pecularities; temporary abstraction of water appears for some reason to render this egg more resistant to the dissolution that otherwise results from the membrane-forming treatment.[2] In other eggs, however, like those of the starfish or *Nereis*, hypertonic sea-water shows no special advantages over a number of other forms of after-treatment. The fact that a double form of treatment has hitherto proved especially effective with the sea-urchin egg is thus not inconsistent with the view that the activation-process is essentially unitary in character in all eggs.

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[1] *Cf.* the experiments summarized in Tables XIII to XVII below.

[2] Compare the experiments of Tables XIV and XV below.

[3] Unpublished experiments performed last summer. An occasional egg may form a larva under this treatment, but the great majority remain unaltered.

[4] In the case of *Strongylocentrotus purpuratus* Loeb found that eggs exposed to butyric acid solutions of the concentrations $n/250$, $n/166$, and $n/125$ for more than 2 minutes failed to form membranes ("Artificial Parthenogenesis and Fertilization," p. 141). Herbst found that eggs of *Sphærechinus* treated for 2, 5, and 8 minutes with a mixture of 50 c.c. sea-water *plus* 3 c.c. $n/10$ acetic acid gave only occasional larvæ (*Roux's Archiv*, 1906, Vol. 22, p. 473). Apparently no systematic experi-

EXPERIMENTAL. EFFECTS OF SIMPLE EXPOSURE TO WARM SEA-WATER.

In these experiments the procedure was similar to that described in my earlier paper.[3] Sea-water at a temperature slightly above that chosen for the experiment was added rapidly to the small beaker containing the eggs (with a thermometer) until the

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ments of this kind have yet been performed with *Arbacia*. At Naples, using *Arbacia pustulata*, Lyon was able to cause development to larvæ in *ca.* 10 per cent. of eggs by exposure to sea-water acidulated with HCl, but he did not try fatty acids (*Amer. Journ. Physiol.*, 1903, Vol. 9, p. 310).

[1] Cyanide is only slightly effective with *Arbacia punctulata* (*cf.* my experiments described in *Journal of Morphology*, 1911, Vol. 22, page 703); it is more so with *Strongylocentrotus*, according to Loeb's results (*cf.* "Artificial Parthenogenesis and Fertilization," p. 80), but even here it is less uniformly favorable than hypertonic sea-water.

[2] *Cf.* the experiments of Loeb (*loc. cit.*, Chapter XI; also *Archiv für Entwicklungsmechanik*, 1914, Vol. 38, p. 409). It is probable that hypertonic sea-water has another and more distinctive mode of action (see below, p. 300).

[3] *Journal of Experimental Zoology*, 1908, Vol. 5, p. 379.

required temperature was reached; this temperature was then kept constant during the period of the experiment by immersing the beaker in a water-bath at the same temperature. At intervals eggs were transferred to sea-water at room-temperature contained in finger-bowls. The exposure to the warm sea-water always took place during the interval between the complete disappearance of the germinal vesicle and the formation of the first polar body.

Exposure to 28°, even if prolonged to 45 minutes, proved almost entirely ineffective in forming membranes in starfish eggs. With exposures of 30 minutes or more an occasional egg may form a membrane, but the great majority always remain unaltered.

At 29° membranes appear in a considerable proportion of eggs after exposures of 12 to 15 minutes. With longer exposures (25 to 30 minutes) a majority in some cases (not always) may form membranes, and a considerable number may develop to larval stages. Table I. summarizes the results of two series of experiments in which eggs were exposed to 29° for periods ranging from 2 to 40 minutes. In both lots of eggs the great majority underwent normal maturation, and a large proportion developed normally to larvæ after sperm-fertilization.

Table I.

29°.

Duration of Exposure in Minutes.	Approximate Proportion of Eggs Forming Fertilization-membranes and Larvæ.			
	Series of June 10.		Series of June 11.	
	Membranes.	Larvæ.	Membranes.	Larvæ.
2 to 10 m.	0	0	0	0
12 m.	*ca.* 2–3%	0	*ca.* 1%	0
14 m.	10– 15%	0	*ca.* 3–4%	0
17 m.	25– 30%	0	*ca.* 10%	0
20 m.	*ca.* 50%	<1%	*ca.* 20%	0
25 m.	70– 80%	*ca.* 2–3%	5–10%	*ca.* 1%
30 m.	30–40%	15–20%	few	*ca.* 5%
40 m.	*ca.* 1–2%	*ca.* 1–2%	0	0

The two series show some minor differences, but in both the number of eggs forming larvæ is small, and a certain proportion fail to form membranes even with the optimal exposures. This temperature is near the lower limit below which the eggs show no response to this form of treatment.

At 30° the proportion of eggs forming membranes and develop- to larval stages is higher than at 29°, although considerable variability is still shown. Five series of experiments were performed at this temperature. Table II. summarizes the results of four of these.[1] Each lot of eggs was favorable, maturation and development to larvæ after sperm-fertilization taking place in nearly all. Table II. gives the approximate proportion of eggs forming membranes and developing to blastulæ after exposure for the periods given in the first column.

TABLE II.

30°.

Duration of Exposure in Minutes.	Proportion of Eggs Forming Fertilization-membranes and Larvæ.							
	June 7.		June 8.		June 12.		June 13.	
	Membranes.	Larvæ.	Membranes.	Larvæ.	Membranes.	Larvæ.	Membranes.	Larvæ.
Up to 3 m.	0	0	0	0	0	0		
4 m.	*ca.* 5%	0	0	0	0	0		
5 m.	15– 20%	0	0	0	0	0	<1%	0
6 m.	*ca.* 20%	0	0	0				
7 m.	*ca.* 30%	0			*ca.* 5%	0	<1%	0
8 m.	*ca.* 80%	0	*ca.* 1%	0			5–10%	0
9–10 m.	*ca.* 100%	*ca.* 2–3 %	20–25%	0	40–50%	0	10–15%	0
12 m.			50–60%	*ca.* 1%	70–80%	0	20–25%	<1%
14–15 m.	*ca.* 100%	*ca.* 5%	80–90%	2– 3%	>90%	10–15%		
17–18 m.			*ca.* 90%	5–10%			30–40%	*ca.* 5%
20–21 m.			<50%	5–10%			*ca.* 40%	*ca.* 30%
24 m.							30–40%	30–40%
28 m.							*ca.* 40%	*ca.* 40%
30 m.			*ca.* 10%	<5%				
34 m.							*ca.* 15–20%	<1%

It will be noted that in four out of the five series at 30° an exposure of 8 to 10 minutes was required to cause membrane-formation in 10 per cent. or more of the eggs; in the fifth series (June 7) 5 minutes was sufficient. The proportion of eggs developing to larvæ was comparatively low in all series; the optimum exposure lay between 24 and 28 minutes in the only series (June 13) in which the proportion of larvæ was considerable. With longer exposures membranes become fewer and there is a rapid decline in the proportion of eggs forming larvæ.

[1] In the remaining series the longest exposure was 10 minutes, at which about two thirds of the eggs formed membranes and a small number developed to larvæ.

At 31° the conditions become more favorable and with the proper times of exposure practically all mature eggs form fertilization-membranes, and in favorable cases the great majority develop to larvæ. Four series of experiments were performed at this temperature; in one of these (August 28) only about half the eggs underwent maturation, and with 15 minutes' exposure (approximately the optimum) only 10 to 15 per cent. of all eggs formed larvæ. In the other three series the eggs were normal. The proportions of eggs forming membranes and larvæ in these series with the different times of exposure are given in Table III.

Table III.

31°.

Duration of Exposures in Minutes.	Proportion of Eggs Forming Fertilization-membranes and Larvæ.					
	June 8.		June 12.		June 13.	
	Membranes.	Larvæ.	Membranes.	Larvæ.	Membranes.	Larvæ.
1–2 m.	0	0	0			
2½ m.			0		0	0
3 m.	0	0	*ca.* 10–15%		*ca.* 5%	0
3½ m.			30– 40%		*ca.* 50%	0
4 m.	Few (<1%)	0	70– 80%		60– 70%	0
5 m.	10–15%	0	*ca.* 90%		>95%	0
6 m.	*ca.* 20%	0	≧90%		*ca.* 100%	0
8 m.	80–90%	*ca.* 2–3%	> 90%	*ca.* 1%	*ca.* 100%	*ca.* 1%
10 m.	*ca.* 90%	15–20%	*ca.* 100%	20–30%	*ca.* 100%	*ca.* 20%
12 m.	*ca.* 90%	40–50%			*ca.* 100%	*ca.* 60%
14–15 m.	70–80%	40–50%			*ca.* 90%	80–90%
17–18 m.	*ca.* 50%	*ca.* 40%			*ca.* 75–80%	50–60%
20–21 m.	15–20%	*ca.* 5%			40–50%	*ca.* 10–15%
25–30 m.					*ca.* 20%	0

At this temperature an exposure of 3 to 4 minutes is required to cause membrane-formation in 10 per cent. or more of the eggs; exposure must be prolonged to *ca.* 8 minutes before any eggs form larvæ; 14 to 15 minutes is the approximate optimum. In the series of June 12 this optimum was not reached.

At 32° a larger number of experiments were performed than at any other temperature, and their results show a decidedly greater uniformity than at lower temperatures. With the optimal times of exposure (from 7 to 8 minutes) the proportion of larvæ yielded by normal eggs is always high,—usually over 90 per cent. This is illustrated by Table IV., which summarizes the results of six successive series performed during June at a time when starfish eggs were unusually abundant and favorable. On account of the relative completeness of my observations at this temperature, the general results of these experiments will be described in some detail.

The exposure required for membrane-formation is about half that at 31°. An exposure of 3 minutes typically forms membranes in all normal eggs, and one of 2 minutes is usually sufficient to produce this effect in a minority and sometimes in a majority of eggs. From 3 minutes on the conditions remain normal for membrane-formation until the exposure is prolonged to 12 or 15 minutes, after which in a certain proportion of eggs membranes tend to separate imperfectly or even fail to form. In most series exposures longer than 15 minutes were not used, since eggs so treated never form larvæ; in one series, however, eggs were exposed for 27 minutes, at which exposure nearly half failed to form membranes. This decline in membrane-formation when exposures are prolonged beyond a certain maximum is general for all temperatures (*cf.* also the series at 30°, 31° and 33°); the fact is interesting since it indicates that the process is not a direct effect of the high temperature but constitutes an active response —probably in the nature of a secretion—on the part of the egg.

The optimum exposure for inducing complete development at 32° varies between 6 and 8 minutes, and with this exposure the great majority of normal eggs cleave and develop to larval stages. Many of the gastrulæ and Bipinnariæ thus obtained are apparently quite normal and swim freely at the surface of the water. The rate of development is, however, always slower than that of sperm-fertilized eggs; relative slowness of development seems in fact to be a constant peculiarity of parthenogenetically activated eggs.[1] Exposures well above the optimum are followed by imperfect or delayed cleavage and failure to develop beyond early stages.

Table IV. gives a summary of the results of the six successive series referred to above. The approximate proportions of eggs forming free-swimming larvæ are given; the conditions of membrane-formation have already been sufficiently described.

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[1] This has been my uniform experience since I began studies of this kind, and apparently the experience is general. This suggests strongly that the spermatozoon contributes to the egg material which is utilized in normal development.

Table IV.

32°.

Time of Exposures.	Proportion of Eggs Forming Free-swimming Larvæ.					
	June 12.	June 13.	June 18.	June 24.	June 25.	June 26.
1–3 m.	0	0	0	0	0	0
4 m.	*ca.* 1%	<1%	*ca.* 4–5%	2– 3%	*ca.* 5%	0
5 m.	*ca.* 3– 4%	2– 3%	15–20%	25–35%	*ca.* 50%	10–15%
6 m.	*ca.* 35–40%	20–30%	55–60%	60–70%	80–90%	25–35%
7 m.		70–80%		≧90%	*ca.* 60%	50–60%
8 m.	>90%	*ca.* 95%	*ca.* 95%	≧90%	25–35%	80–90%
10 m.	85–90%	50–55%	75–85%	50–60%	< 5%	80–90%
12 m.		15–20%	25–35%		0	*ca.* 20%
15 m.		0	<1%			
18, 22, and 27 m.		0	0			

These results may safely be regarded as typical. Six other similar series were carried out at this temperature. In two of these the eggs were unfavorable or the treatment was applied too late. In the four others—two in early June and two in late August—the results were similar to the above, although fewer eggs formed larvæ; the optimum exposures ranged from 6 to 8 minutes, with respectively 20, 20, 40 and 50 per cent. of mature eggs forming larvæ. It will be noted that the optimum exposure is approximately 8 minutes in five out of the six series in Table IV. Different lots of eggs vary somewhat in the duration of this optimum; thus in the series of June 25 half of the eggs formed larvæ with only five minutes' exposure and the optimum was 6 minutes, and on June 26 the eggs showed almost equally good development with the 8- and the 10-minute exposures. In the majority of series, however, there was a well-defined optimum at 7 or 8 minutes.

The physiological effects following exposure to 32° vary in a constant and highly characteristic manner with the duration of the exposure. Eggs exposed for a period insufficient to induce membrane-formation show no apparent change on return to sea-water and later break down without development. Such eggs, however, can be shown to have undergone some internal change similar in kind to that following longer exposures; thus if later they are again exposed to 32° they are found to require, in order to induce favorable development, a shorter exposure than previously untreated eggs (*cf.* below, p. 288). Exposure for 3 to 4 minutes induces typical membrane-formation in all eggs, followed, however, not by cleavage and further development but by irregular changes of form, fragmentation, and eventual breakdown. With somewhat longer exposures (4 to 5 minutes) membrane-formation is followed by symmetrical cleavage in a certain proportion of eggs; and the proportion of such cleavages, and also their approximation to the normal in rate and character, show a progressive increase with increasing length of exposure up to the optimum of about 8 minutes. With still longer exposures the response again becomes unfavorable, and eventually the eggs entirely fail to develop and even to form membranes. We have here an apparent reversal of the rule enunciated by Loeb with reference to the action of membrane-forming agents on the sea-urchin egg. "A relatively brief exposure to a cytolytic agent leads only to membrane-formation, while a longer exposure causes cytolysis."[1] In the starfish egg a relatively brief exposure to warm sea-water (one just sufficient for membrane-formation) is followed by an early cytolysis, while a longer exposure results not only in membrane-formation but in an approximately normal development; still longer exposures again cause cytolysis without development. This rule applies to the action of cytolytic substances like fatty acid, as well as to high temperatures (*cf.* below, p. 282).

To illustrate the effects of exposures of different duration on cleavage the following record is given (Table V.) describing the condition of the eggs about four hours after exposure to 32° for the times given.

It will be noted that with brief exposures (3 to 4 minutes) membrane-formation is typical, but the eggs are unable to cleave normally and undergo irregular change of form followed by breakdown. As the time of exposure increases, an increasing proportion of eggs cleave, until the optimum (6 to 7 minutes) is reached at which cleavage approaches the normal in rate and character, and the great majority develop to larval stages. Over-exposure (10 minutes) is again followed by failure of cleavage and development. Similar observations were made in experiments at other temperatures; in all cases the exposure which induced the largest proportion of regular cleavages was found to correspond with that at which the largest proportion of eggs formed larvæ. In the series of Table V. the optimum, 6 minutes, is somewhat shorter than usual; on June 24 the optimum of cleavage was found at 7 minutes, and on June 26 at 8 minutes, with 10 minutes somewhat less favorable.

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[1] "Artificial Parthenogenesis and Fertilization," 1913, p. 8.

TABLE V.

JUNE 25. 32°.

Time of Exposure.	Condition of Eggs 4 Hours after Exposure, and Proportion of Eggs forming Larvæ.
1. 2 m.	Great majority are unchanged; a few have membranes. No larvæ.
2. 3 m.	Most eggs have typical membranes and are irregular or amœboid in form; a few show irregular cleavages. No larvæ.
3. 4 m.	Almost all eggs have membranes and exhibit irregular forms; a few have cleaved symmetrically. *Ca.* 5 per cent. form larvae.
4. 5 m.	Marked improvement over Experiment 3: most eggs have cleaved, and many are in regular 4- and 8-cell stages. *Ca.* 50 per cent. form larvæ.
5. 6 m.	Almost all eggs are cleaved; cleavages are more regular and advanced than in Exp. 4; 16-cell stages are frequent. 80–90 per cent. form larvæ.
6. 7 m.	The proportion of regular cleavages is also high, but rather less than in Exp. 5. *Ca.* 60 per cent. form larvæ.
7. 8 m.	Cleavages are fewer and less advanced than in Exp. 6. *Ca.* 25–30 per cent. form larvæ.
8. 10 m.	Great majority are uncleaved; many are irregular in form or fragmented. Larvæ are few: < 5 per cent.
9. 12 m.	Almost none have cleaved. The eggs are largely irregular or with small surface-vesicles detached.

While an exposure just long enough for membrane-formation is insufficient by itself to induce normal cleavage and development, it is possible, after forming membranes in this way, to make the eggs cleave and develop to larval stages by subjecting them to a second treatment with warm sea-water, or by after-treatment with fatty acid ($n/260$ butyric acid in sea-water), hypertonic sea-water, or cyanide ($n/1000$ KCN in sea-water). These effects will later be described in detail (*cf.* Tables XIII., XVII.).

It is interesting to note that the effects produced by weak fatty acid solutions ($n/260$ butyric acid) on unfertilized starfish eggs also vary with the time of exposure in a manner closely similar to that just described. Brief exposure causes membrane-formation followed by irregular change of form and breakdown without development, while longer exposure induces not only membrane-formation but cleavage and development to larval stages; still longer exposure is again unfavorable. Eggs in which membranes have been formed by the minimal exposure to fatty acid may be made to develop by the above forms of after-treatment. The effects of the two agents, warm sea-water and weak butyric acid solution, seem in fact to be identical in every essential particular, and the one may be substituted for the other without altering the effect on the egg (*cf.* Tables XIV. to XVII.). Experiments showing this parallelism will be described in detail later. There is in fact every indication that the underlying physiological process which enables the egg to continue normal development is of the same nature as that which induces simple membrane-formation, the only difference being that the duration of the process must be considerably longer in the second case than in the first. The temperature-coefficients of both effects indicate the same, as will appear below (*cf.* Table X.). The possible nature of this process will be discussed in the concluding section of this paper.

Treatment with sea-water at 33° gives similar results to those above described, except that the times required to produce a given physiological effect are only a little more than half as long as at 32°. An exposure of from one to one and a half minutes is needed to call forth membrane-formation in the majority of eggs. Four series of experiments with normal eggs were performed at this temperature, and in every series the great majority of eggs formed larvæ with the optimal times of exposure. In these series the earlier transfers from the warm sea-water to normal temperature were made at half-minute intervals. The results are summarized in Table VI.

In all of these series the proportion of favorably developing eggs is high with the optimum exposures of 4½ to 5½ minutes. The series of June 15 is unusual in that nearly all of the eggs form larvæ with exposures varying in length from 4 to 6 minutes. The optima seem to be less sharply defined when the eggs are in the best of condition, probably because then the power of regulatory adjustment to environmental variations is at its maximum, and slight deviations from the optima are automatically corrected.

Table VI.

33°.

Time of Exposure.	Proportion of Eggs Forming Larvæ (Blastulæ and Gastrulæ).			
	June 9.	June 10.	June 15.	June 17.
1–1½ m.	0	0	0	0
2 m.	0	Very few (<0.1%)	0	0
2½ m.	Few (<1%)	*ca.* 1%	< 1%	
3 m.	10–15%	*ca.* 10%	*ca.* 5%	25–30%
3½ m.	*ca.* 50%	50–60%	20–30%	
4 m.	80–90%	80–90%	*ca.* 90%	55–65%
4½ m.	60–70%	⋝90%	*ca.* 90%	
5 m.	40–50%	90–95%	*ca.* 95%	90–95%
5½ m.			90–95%	
6 m.	20–25%	>90%	*ca.* 90%	*ca.* 95%
7 m.	*ca.* 2–3%		75–85%	
8 m.		70–80%	10–15%	70–80%
10 m.		15–20%	0	30–40%
12 m.			0	
15, 18, 21 and 25 m.			0	

At 34° the majority of eggs form membranes with one minute's exposure, and 30 seconds is sufficient for a minority. A few eggs form larvæ after 2 minutes' exposure; the optimum is 3 to 4 minutes; longer exposure is injurious. Table VII. summarizes

Table VII.

34°.

Time of Exposure.	Proportion of Eggs Forming Larvæ.			
	June 10.	June 15.	August 26.	August 27.
30, 1 m.	0	0		
1½ m.	*ca.* 1%	0		
2 m.	25–35%	0	0	*ca.* 1%
2½ m.	50–60%	20–30%	*ca.* 1%	
3 m.	65–75%	*ca.* 90%	5–10%	25–30%
3½ m.	70–80%	*ca.* 90%	10–15%	
4 m.	50–60%	*ca.* 90%	15–25%	35–40%
4½ m.		*ca.* 90%	20–30%	
5 m.	5–10%	*ca.* 10%	20–30%	*ca.* 5%
5½ m.		*ca.* 1%		
6 m.	<1%	0	10–15%	0
7, 8, 10 and 12 m.	0	0	0	

the results of four series of experiments at this temperature. The August eggs were less favorable than the June eggs.

Two similar series at 35° and one at 36° were carried out in June at a time when starfish eggs were unusually favorable. The results were similar to those at 33° and 34° except that the physiologically equivalent exposures were shorter. At 35° an exposure of 30 seconds induces membrane-formation in many but not in all eggs, and one of 45 seconds in practically all. At 36° 15 seconds is sufficient to form membranes in about half the eggs and 30 seconds in all. Longer exposures eventually interfere with membrane-formation; thus after 6 to 8 minutes at 36° membranes failed to form or were imperfect in 40 to 50 per cent. of eggs. Table VIII. gives the proportions of eggs forming swimming larvæ in these experiments. The transfers from warm to normal sea-water were made at first at intervals of fifteen seconds.

Table VIII.

35° and 36°.

Time of Exposure.	Proportion of Eggs Forming Larvæ.		
	June 15 (35°).	June 16 (35°).	June 17 (36°).
15″, 30″	0	0	0
45″	0	0	20–30%
1 m.	*ca.* 1%	0	85–90%
1 m. 15″	*ca.* 5%	*ca.* 4–5%	⋝95%
1 m. 30″	80–90%	35–45%	70–80%
1 m. 45″	>95%	70–80%	*ca.* 10%
2 m.	*ca.* 90%	⋝90%	none free
2½ m.	*ca.* 50%	40–50%	0
3 m.	*ca.* 5%	5–10%	0
3½ m.	0	0	0
4 m.–10 m.	0	0	0

The rapid decrease in the optimum exposures as the temperature rises is to be noted; the optima are respectively 1½ to 2 minutes at 35°, and 1 to 1¼ minutes at 36°.

Variation with Temperature in the Rate of the Process Underlying Activation by Heat.

The foregoing results show that the process, whatever its nature, which is initiated in the mature unfertilized starfish egg by temperatures of 30° to 36°, and which brings the egg into a condition to form membranes cleave, and develop, proceeds twenty or twenty-five times as rapidly at 35° as at 30°. Thus at 30° the minimum exposure for membrane-formation is about 10 minutes, and at 35° 30 seconds or less; similarly at 30° the minimum exposure needed to induce even a few eggs to form larvæ is about 20 minutes, at 35° it is about 1 minute; at 30° the optimum exposure is *ca.* 30 minutes, and at 35° *ca.* 1½ minutes. For each temperature it is possible to assign a definite length of exposure which produces a definite effect on the egg. The manner in which these times of exposure vary at different temperatures may be seen by reference to Table IX. Here are

TABLE IX.

Temperature and Series.	Minimum for Membrane-formation.	Minimum for Larvæ.	Optimum for Larvæ.	Maximum for Larvæ.
29° (June 10)	14 m.	25 m.		
30° (June 13)	8–10 m.	12–18 m.	24–28 m.	
31° (June 8)	5 m.	8 m.	12–14 m.	20 m.
(June 12)	3 m.	8 m.		
(June 13)	3½ m.	8 m.	14–15 m.	21 m. (<25 m.)
32° (June 12)	2½ m.	4 m.	8 m.	
(June 13)	2 m.	4–5 m.	8 m.	12 m. (<15 m.)
(June 18)	2 m.	4 m.	8 m.	12 m. (<15 m.)
(June 24)	2–3 m.	4 m.	7–8 m.	? (>10 m.)
(June 25)	2 m.	4 m.	6 m.	10 m. (<12 m.)
(June 26)	3 m.	5 m.	8–10 m.	12 m.
33° (June 9)	2 m.	3 m.	4 m.	7 m.
(June 10)	1 m.	2½ m.	4½–6 m.	10 m.
(June 15)	1 m.	3 m.	4–6 m.	8 m. (<12 m.)
(June 17)	1 m.	3 m.	5–6 m.	10 m.+
34° (June 10)	30 sec.	1½ m.	3–3½ m.	5 m. (<6 m.)
(June 15)	1 m.	2½ m.	3–4 m.	5½ m. (<6 m.)
35° (June 15)	30 sec.	1 m.	1 m. 30″–1 m. 45″	3 m. (<3½ m.)
(June 16)	30 sec.	1 m. 15″	*ca.* 2 m.	3 m. (<3½ m.)
36° (June 17)	15 sec.	45″(>30″)	1 m.–1 m. 15″	*ca.* 2 m. (<2½ m.)

tabulated the observations made in all of those series of experiments in which a large proportion of eggs formed larvæ,—in which, therefore, the conditions may be regarded as essentially normal. In the series at 29° few eggs formed larvæ; at 30° only one series out of five gave a considerable proportion of larvæ (*ca.* 40 per cent.) with *ca.* 30 minutes' exposure; in all of the other series in the table, except one at 31°, the great majority of eggs—usually over 90 per cent.—formed larvæ with the optimal exposures. In the first column is given the least time of exposure required for membrane-formation in a significant proportion of eggs—10 per cent. or more; in the second column the least exposure at which any eggs (> 1 per cent.) formed larvæ; in the third the optimal time of exposure; and in the fourth the longest observed exposure at which any eggs (> 1 per cent.) formed larvæ.

If the several observed durations at each temperature are averaged, the following results are obtained (Table X.); the values are given in approximate terms rather than strict arithmetical averages, to emphasize the fact that the precise durations vary to a certain degree, even in normal eggs. There is, however, for each temperature a well-defined modal duration of exposure for producing a definite physiological effect such as membrane-formation or complete activation.

TABLE X.

APPROXIMATE TIMES OF EXPOSURE REQUIRED TO PRODUCE THE FOLLOWING EFFECTS AT DIFFERENT TEMPERATURES.

Temperature.	Formation of Membranes.	Minimum for Larvæ.	Optimum for Larvæ.	Maximum for Larvæ.
29°	*ca.* 12–14 m.	20–25 m.	30–40 m.	
30°	8–10 m.	*ca.* 18 m.	*ca.* 28 m.	? >30 m.
31°	*ca.* 4 m.	*ca.* 8 m.	*ca.* 15 m.	21–25 m.
32°	*ca.* 2 m.	4– 5 m.	7– 8 m.	10–12 m.
33°	*ca.* 1 m.	$2\frac{1}{2}$– 3 m.	$4\frac{1}{2}$–$5\frac{1}{2}$ m.	8–10 m.
34°	30″ to 1 m.	*ca.* 2 m.	3–$3\frac{1}{2}$ m.	*ca.* 5 m.
35°	*ca.* 30″	1–$1\frac{1}{4}$ m.	$1\frac{1}{2}$–$2\frac{1}{2}$ m.	*ca.* 3 m.
36°	*ca.* 15″	30″–45″	1–$1\frac{1}{4}$ m.	*ca.* 2 m.
37°			30–35 sec.	
38°			*ca.* 20 sec.	

It will be noted (1) that for each temperature there is a minimum effective exposure which induces membrane-formation followed by failure to develop and early breakdown; and (2) that an exposure of approximately twice the minimum for membrane-formation is required to enable even a few eggs to develop to larval stages, and an exposure of three or four times this minimum to enable development to proceed normally in all eggs; and (3) that if the exposure is prolonged to about one and a half times this optimum the eggs are again incapacitated from further development. The fact that the ratios of the durations required to produce these several effects are approximately the same at any one temperature indicates that a single process of a definite kind forms the determining condition of all. This process is peculiar in undergoing marked acceleration by slight rise of temperature; it is also clear, from the fact that an effective exposure must last for a certain minimal time at any temperature, that the process must proceed to a definite stage before the egg is rendered capable of continuing its development to advanced stages; if the process is arrested before its completion, only the earlier developmental changes can be carried out (membrane-formation, early cleavage or change of form); if, on the other hand, it is allowed to proceed too far, injurious conditions arise which eventually prevent all development; a sufficiently prolonged exposure to high temperature renders the egg incapable even of membrane-formation.

In endeavoring to form some consistent conception of the nature of this process the following facts have to be considered. It exhibits a high temperature-coefficient: from fifteen to twenty times the duration of exposure is required to induce membrane-formation at 30° as at 35°; the ratios between 29° and 34° and between 31° and 36° are the same. At each temperature the proportionate durations of the minimum, optimum, and maximum exposures for forming larvæ are approximately the same. In other words, the critical change underlying simple membrane-formation is affected by temperature in the same way as that underlying complete activation of development: *i. e.*, the proportionate increase in velocity by rise of temperature is the same in both cases, a fact which can only indicate that one fundamental process—and not two—is concerned in producing both effects. If we assume that the above proportionate increase in velocity prevails through a rise of 10°, a Q_{10} value of from 225 to 400 is indicated, as against the 2 to 3 characteristic of chemical reactions in homogeneous media.[1] Activation by heat thus depends on some critical change in the egg which does not begin until a temperature of about 29° is reached, but which undergoes very rapid acceleration with further rise of temperature. The liquefaction of gels by heat seems to be the only relevant process which shows these characteristics. The change in viscosity preceding the gelation of a gelatine sol undergoes very rapid acceleration with lowering of temperature, within a few degrees of the temperature of gelation. The inverse process, melting of gels, has a similarly high temperature-coefficient (*cf.* below, p. 295). In general the facts suggest that the direct effect of the high temperature is to cause a change in the colloidal system of the egg, of such a kind as to render possible a chemical interaction between substances which in the normal condition of the resting egg are kept apart. This restraining condition may be some physical barrier like a membrane, impermeable to the diffusion of the substances concerned, or it may be a certain state of electrical polarization of the general cell-surface, as suggested below (p. 299). It is also important to note that the activation-process may be arrested by a return of the eggs to sea-water at ordinary temperatures, and renewed after an interval without interfering with its effect. A reversibility of the physico-chemical change forming its basis is thus indicated. It should further be noted that cytolytic agents like butyric acid not only have the same general physiological effect as brief warming, but that the relations between time of exposure and physiological effect produced are the same in both cases. Some process which is affected similarly by these two dissimilar agents is thus to be sought. In the following section the results of experiments with weak butyric acid solution are described in greater detail.

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[1] The temperature-coefficients of the rate of cytolysis of sea-urchin eggs and of the duration of life of sea-urchin larvæ and of Tubularia stems at temperature of 25° to 40° show similarly high values. In these cases the direct effect produced on the cell by the high temperature is probably of the same kind as that underlying the above activation-effect; this appears to be a change in the colloids of the plasma-membranes, leading to an increase of permeability. (See below, p. 296.) Such a change if not reversed within a certain time results in cytolysis. In the unfertilized starfish egg *temporary* increase of permeability involves activation. For data on the temperature-coefficients of cytolysis and heat-death, *cf.* J. Loeb,

EFFECTS OF EXPOSURE TO BUTYRIC ACID SOLUTION FOR DIFFERENT PERIODS.

As already stated, treatment of starfish eggs during the early maturation period with weak solutions of butyric acid in sea-water (*n*/260) produces the same effects as temporary warming,

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Archiv f. d. ges. Physiologie, 1908, Vol. 124, p. 411; A. R. Moore: *Quarterly Journal of Experimental Physiology*, 1910, Vol. 3, p. 257; *Arch. f. Entwicklungsmech.*, 1910, Vol. 29, pp. 146, 287.

and the time-relations of the exposures necessary for these effects are closely similar with both methods. Table XI. summarizes the results of five series of experiments with separate lots of eggs. The eggs were exposed at normal temperatures (20° to 22°) to an *n*/260 solution of butyric acid in sea-water (50 c.c. sea-water *plus* 2 c.c. *n*/10 butyric acid), and portions were transferred to normal sea-water at the intervals named. The approximate proportion of mature eggs developing to free-swimming larvæ (blastulæ and gastrulæ) is given.

TABLE XI.

N/260 BUTYRIC ACID.)

Time of Exposure.	Proportion of Mature Eggs Developing to Larvæ.				
	Series 1 (Aug. 31.)	Series 2 (Sep. 1).	Series 3 (Sep. 1).	Series 4 (Sep. 2.)	Series 5 (Sept. 2).
1 m.	0	0	1 or 2 larvæ	1 blastula	<1%
2 m.	2 or 3 larvæ	0	*ca.* 1%	*ca.* 1%	2–3%
3 m.	<1%	<1%	*ca.* 4–5%	1– 2%	20–30%
4 m.	<1%	*ca.* 1%	*ca.* 10%	5–10%	55–60%
5 m.	*ca.* 1%	*ca.* 10%	10–15%	20–30%	75–85%
6 m.	*ca.* 5%	*ca.* 50%	20–30%	30–40%	80–90%
7 m.	20–30%	70–80%	40–50%	20–25%	35–40%
8 m.	*ca.* 50%	65–75%	50–60%	15–20%	20–30%
10 m.	80–90%	65–75%	*ca.* 60%	*ca.* 1%	10–15%
12 m.	30–40%	25–35%	40–50%	1 blastula	0
15 m.	<1%	*ca.* 1%	20–30%	0	0

The close parallelism between these experiments and those of warming to 32° or 33° will at once be noted. With brief exposure there is the same simple membrane-formation followed by breakdown without development; as the exposure is prolonged there is a progressive increase in the proportion of favorably developing eggs up to an optimum; then follow a decrease and eventual failure to develop. More detailed observations show that the rate and regularity of cleavage show a corresponding steady improvement up to an optimum which is again followed by a decline.

The following observations show the condition of the eggs in the second series of September 1, at about four hours after the treatment with butyric acid (Table XII.).

The optimum time of exposure shows somewhat more variability in these series than is usually the case with exposure to warm sea-water (32°); in all five, however, the optimum lay between five and ten minutes.[1] There is thus an approximate constancy in the time of exposure required to induce complete development with solutions of this concentration. Probably

TABLE XII.

N/260 BUTYRIC ACID.

Time of Exposure.	Condition of Eggs 4 Hours after Treatment, and Proportion forming Larvæ.
1 m.	All eggs have membranes; most are irregular or amœboid in form; none are cleaved. No larvæ.
2 m.	Similar to 1 m. lot, but a few eggs (*ca.* 2–3 per cent.) are in the 2-cell stage. No larvæ.
3 m.	Generally similar to the 2 m. lot, but the cleavages are more numerous (*ca.* 10–15 per cent.), mostly 2-cell with a few 4-cell stages. Very few larvæ (< 1 per cent.).
4 m.	Cleavages are more numerous and advanced; 40–50 per cent. are cleaved, mostly 2 and 4-cell, with a few 8-cell stages. Larvæ still few (*ca.* 1 per cent.).
5 m.	Cleavage is more advanced than in the 4 m. lot; *ca.* 50 per cent. are cleaved, largely 8- and 16-cell stages. *Ca.* 10 per cent. form larvæ.
6 m.	Most eggs are cleaved (*ca.* 70–80 per cent.), many in 16- to 32-cell stages. *Ca.* 50 per cent. form larvæ.
7 m.	Almost all eggs are cleaved (90 per cent. or more), many in normal-looking 16- to 32-cell stages. 70–80 per cent. form larvæ.
8 m.	Similar to 7 m. lot; most eggs are in 16- to 32-cell stages. 65–75 per cent. form larvæ.
10 m.	Cleavages are fewer and less advanced; *ca.* 70–75 per cent. are cleaved, mostly 4- and 8-cell stages. 65–75 per cent. form larvæ.
12 m.	Comparatively few cells are cleaved; *ca.* 10 per cent. are in 2- or 4-cell stages, largely irregular; the rest uncleaved. 25–35 per cent. form larvæ.
15 m.	Almost all eggs remain uncleaved, and many show the beginnings of surface-disintegration. Few form larvæ,—*ca.* 1 per cent.

Controls: Unfertilized eggs disintegrate without membrane-formation or development. Nearly all sperm-fertilized eggs develop to larvæ.

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[1] This variability may be due partly to the fact that on account of the lateness of the season and consequent scarcity of ripe starfish the eggs used in these experiments came from fewer animals; thus in Series 1, 4, and 5, eggs from only one starfish were used in each case, and in Series 2 and 3 from three. In the earlier experiments with warm sea-water the mixed eggs from several animals were used in each series.

an inverse relation exists between the concentration of fatty acid and the time of exposure required to produce a given effect. Systematic experiments to determine the character of this relation have not yet been carried out, but there are some observations bearing on this question. In several of my experiments in the summer of 1912 starfish eggs exposed for only one minute to acetic or butyric acid of *ca.* *n*/176 concentration (6 c.c. *n*/10 acid *plus* 100 c.c. sea-water) formed a large proportion of larvæ.[1] Lyon observed some years ago that the exposure required to induce parthenogenesis in *Arbacia pustulata* by means of weak solutions of HCl in sea-water decreased with increase in the concentration of acid up to a certain point.[2] The minimum exposure to *n*/260 butyric acid required to form membranes is very brief in starfish eggs. Experiments last summer showed that while 10 seconds was insufficient to form membranes in more than a few eggs (*ca.* 10 per cent.), with 20 seconds all formed membranes, followed by the typical irregular changes of form and breakdown. After one minute's exposure to *n*/260 butyric acid an occasional egg may form a blastula; yet in the series showing the shortest optimum exposure of any performed last summer (No. 5, Sept. 2) at least 3 minutes was required to enable any considerable proportion of eggs to develop to a larval stage. The parallelism between the effects of high temperature and of weak fatty acid solutions indicates that the two agents act by producing the same kind of change in the egg-system. More detailed experiments to determine the influence of concentration as well as time on the action of this and other cytolytic substances remain to be carried out, and their results will probably throw further light on the nature of this change.

Effects of Membrane-formation by Heat or Fatty Acid Combined with After-treatment by the Same Agent.

The fact that a longer treatment with the membrane-forming agent produces the same effect as a short treatment combined with after-exposure to hypertonic sea-water or cyanide suggests that a suitable after-treatment with the membrane-forming agent itself should have a corrective effect similar to that exerted by the agents just named. If the effect of the initial or membrane-forming treatment is to cause a partial activation which requires later to be completed by the after-treatment, we should expect it to be a matter of indifference (within certain limits of time) whether the activation is completed in one stage—*e. g.*, by a continuous warming to 32° for 8 minutes—or in several; development ought to follow equally well if the eggs are returned to sea-water after an exposure just sufficient for membrane-formation, and afterward again exposed to the same treatment for an appropriate length of time. Experiment shows that it is in fact possible to substitute for the after-treatment with hypertonic sea-water or cyanide a brief exposure either to warm sea-water or to *n*/260 butyric acid. We have here clear indication that the essential changes produced in the egg by after-treatment with an agent like hypertonic sea-water are not qualitatively different from those caused by the first or membrane-forming treatment, but serve simply to renew and bring to its completion a process which has been initiated by the first treatment but prematurely arrested by the early return to normal sea-water. According to this conception the whole activation-process is unitary in nature and does not consist of two qualitatively distinct and mutually complementary processes, as Loeb has maintained on the basis of his experiments with sea-urchin eggs.

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[1] Cf. *Journal of Experimental Zoölogy*, 1913, Vol. 15, pp. 41, 42.

[2] Lyon, *American Journal of Physiology*, 1903, Vol. 9, p. 310.

The following series (Table XIII.) illustrates the effects of treating eggs, in which membranes have been formed by 3 minutes' exposure to 32°, a second time with sea-water at 32° for 4 minutes; the second exposure was made at varying intervals after the first, ranging from 9 minutes to nearly 4 hours.

Table XIII.

After-treatment with Sea-water at 32°.

June 24. Eggs from several starfish were exposed, about 35 minutes after removal from the animals, to sea-water at 32° for 3 minutes (11.12–11.15 A.M.), and then returned to sea-water. Part of these eggs were left permanently in sea-water for control; the rest were again exposed to 32° for 4 minutes, successive portions being thus treated at 10-minute intervals until well after the separation of the second polar body. The condition of the maturing eggs at the time of the second treatment is indicated in the first column.

Control lots of eggs were exposed (for purposes of comparison) to 32° for single continuous periods ranging from 2 minutes to 10 minutes.

After-exposures to 32° at Following Times after First Exposure.	Results (Condition of Eggs *ca.* 4 Hours Later, and Proportion Forming Larvæ.
1. Control: no second exposure	All mature eggs have membranes but are uncleaved and largely irregular in form. None form larvæ.
2. 9 m. (11.24–11.28) (no polar bodies at 11.28)	Marked contrast to control; almost all eggs are cleaved, largely to 32- or 64-cell stages. *Ca.* 70–80 per cent. form larvæ many of which swim at the surface of the water.
3. 19 m. (11.34–11.38) (first polar bodies beginning to separate at 11.38)	Cleavage is less advanced than in Experiment 2, and a minority are uncleaved. Somewhat fewer larvæ (*ca.* 65–75 per cent.).
4. 29 m. (11.44–11.48) (first polar bodies in all eggs at 11.48)	Cleavages are fewer and less advanced than in Experiment 3. Most eggs are uncleaved. Larvæ fewer (*ca.* 30–40 per cent.).
5. 39 m. (11.54–11.58) (all with first polar bodies, none with second at 11.58)	Contrast to Exp. 4; great majority are uncleaved and largely irregular; a few 2- and 4-cell stages present; few form larvæ (*ca.* 5 per cent.).
6. 49 m. (12.04–12.08) (*ca.* 50 per cent. have second polar body at 12.08)	Similar to Exp. 5 but with fewer cleavages. Larvæ also are fewer (*ca.* 2–3 per cent.).
7. 59 m. (12.14–12.18) (all eggs have second polar bodies)	Nearly all are uncleaved; largely irregular or fragmented. Almost no larvæ (only one feeble blastula seen).
8. 1 h. 9 m. (12.24–12.28)	All are uncleaved but irregular forms are fewer. No larvæ.
9. *ca.* 3 h. (2.59–3.03)	Similar to Exp. 8.

Controls with one exposure to 32°: eggs exposed 7 minutes continuously (11.12–11.19) gave *ca.* 90 per cent. larvæ. With 4 minutes' exposure few eggs (*ca.* 2–3 per cent.) formed larvæ. Controls of unfertilized and sperm-fertilized eggs were normal.

Experiments similar to the above were performed with preliminary exposures to 32° of 2, 3 and 4 minutes, followed by after-exposure to 32° as above for 4 minutes (in one series for five minutes), all of which gave the same general result. Apparently it is a matter of indifference whether the second exposure to 32° follows immediately after the first or at an interval, provided that the second exposure takes place before the separation of the first polar body. After this event there follows a decided and rapid decline in the favorability of the response to the after-warming treatment, and after the separation of the second polar body after-warming is apparently quite ineffective. As I described in my former paper on this subject, the susceptibility to parthenogenesis by temporary continuous warming always undergoes marked and rapid decrease at the time of the maturation-divisions.[1] The above decline in the response to after-warming is evidently the same phenomenon. A similar decrease in the susceptibility of the eggs to sperm-fertilization also takes place at about the same time, although this decrease is not so pronounced as in the case of parthenogenesis; thus it is usually possible to fertilize a certain variable proportion of starfish eggs (not all) after maturation has been complete for some hours.[2] The fact that the general responsiveness of the egg to any activating agent undergoes a sudden decline at the time of separation of the polar bodies suggests either that some material necessary to development is then lost, or that a refractory state conditional on some other kind of change (possibly a change in the plasma-membrane) then develops. As already pointed out, the fact that sperm-fertilization is possible (although less favorable) at a time when the egg fails to respond to the parthenogenetic treatment suggests that some definite material playing an important part in development is introduced into the egg by the sperm. This is also indicated by the general fact that sperm-fertilization induces a more favorable development than artificial activation. It may be that this material is the same as some substance lost from the egg at the time of the maturation-divisions. Further research has to decide between these possibilities.

[1] *Loc. cit.*, 1908, p. 400.

[2] *Loc. cit.*, p. 411.

In the above described experiments the total optimum period of exposure to 32° is about the same (*ca.* 7 to 8 minutes) whether the exposure is continuous or in two stages. No doubt it would be possible to increase the number of stages to three or more, especially if lower temperatures (31° or 30°) were used, but no experiments of this kind have so far been attempted. Apparently what is essential is that the critical process begun by the warming should continue, at the given temperature, for a certain definite length of time, sufficient presumably to allow some critical chemical interaction to proceed to its completion. It is interesting to note that a preliminary warming which is too brief in itself to cause membrane-formation may nevertheless have the effect of shortening the period of after-warming necessary to cause complete development. In one experiment the preliminary exposure to 32° was only 2 minutes, a time insufficient for membrane-formation in more than very few eggs (*ca.* 1 per cent.); these eggs, however, when again exposed to 32° for 4 minutes, gave a considerable proportion of larvæ (5 to 10 per cent.); while eggs exposed to 32° for 4 minutes without any previous treatment formed membranes, but none developed to larvæ. A continuous single exposure of 6 minutes gave 25 to 35 per cent. of larvæ; this exposure was well below the optimum of 8 to 10 minutes at which 80 to 90 per cent. formed larvæ. This effect of the four minutes' after-exposure on eggs which otherwise showed no external change indicates that membrane-formation is not in itself a critical event, but simply an expression of a partial initiation of the general developmental process: *i. e.*, a partial activation has been accomplished, enabling the egg to carry out a few of the early steps in development.

Since brief exposure to weak fatty acid solution has the same physiological effect on the egg as brief warming, it would appear that the essential change produced in the egg-protoplasm by either form of treatment is the same; if so, after-treatment with warm sea-water should have a similarly favorable effect on eggs in which membranes were formed by fatty acid. The following series of experiments shows that this is the case (Table XIV.). The eggs, after membrane-formation by butyric acid, were after-treated with warm sea-water (32°) for periods ranging from 2 to 12 minutes. For comparison part of the eggs were after-treated with hypertonic sea-water and cyanide.

Table XIV.

n/260 Butyric Acid with After-treatment with Sea-water at 32°.

August 24. Eggs from one starfish were used. These eggs were not very favorable and a rather small proportion underwent maturation. They were exposed, about 45 minutes after removal, to *n*/260 butyric acid solution for one minute and then returned to normal sea-water. Twelve to sixteen minutes later they were after-treated as follows, with the results indicated.

After-treatment.	Results (Condition of Eggs 4–5 hours later, and Proportion of Mature Eggs forming Larvæ).
1. None (control treated with butyric acid alone)	Typical fertilization-membranes in all mature eggs; later the eggs assume irregular forms and break down. None form larvæ.
2. Hypertonic sea-water (250 c.c. s. w.+40 c.c. 2.5 m NaCl) for 30 min.	Markedly favorable effect: most mature eggs are cleaved to *ca.* 32-cell stage. 20–30 per cent. form larvæ.
3. M/1000 KCN in sea-water for 30 min.	Eggs cleave as in Experiment 2. *Ca.* 25–30 per cent. form larvæ.
4. Sea-water at 32° for 2 min.	After four hours most eggs are irregular and uncleaved; a few are cleaved. Very few form larvæ (< 1 per cent.).
5. 32° for 3 min.	Like Exp. 4, but more eggs are cleaved. Few larvæ, —1 per cent. or less.
6. 32° for 4 min.	Cleavages are more numerous than in Exps. 4 and 5. *Ca.* 5 per cent. of mature eggs form larvæ.
7. 32° for 5 min.	*Ca.* 20–30 per cent. are cleaved. *Ca* 5 per cent. form larvæ.
8. 32° for 6 min.	Cleavages are more numerous than in Exp. 7. *Ca.* 40–50 per cent. larvæ.
9. 32° for 7 min.	Most eggs in 16- to 64-cell stages. 50–60 per cent. form larvæ.
10. 32° for 8 min.	Like Exp. 9, but somewhat less favorable. *Ca.* 50 per cent. form larvæ.
11. 32° for 10 min.	After five hours few eggs are cleaved and cleavages are less advanced than in Exps. 9 and 10. *Ca.* 10–15 per cent. form larvæ.
12. 32° for 12 min.	Almost none have cleaved after five hours. Practically none form larvæ (one blastula seen).

For comparison eggs were exposed to 32° without previous membrane-formation for 4, 5, 6, 7, 8, and 10 minutes; the optimum exposure was 8 minutes at which 50–60 per cent. of the mature eggs formed larvæ. A sperm-fertilized control also yielded numerous larvæ.

After-exposure to 32° for the proper time thus greatly increases the proportion of favorably developing eggs. No marked improvement is seen until the duration of after-exposure reaches four minutes; with longer exposures the proportion of eggs forming larvæ shows progressive increase up to an optimum at about seven minutes; a decline then follows; an exposure of 10 minutes effects only slight improvement, and one of 12 minutes appears ineffective. Similar results, differing slightly in detail in different series, were obtained in eight other series of experiments. In general, after the preliminary membrane-formation by one minute's exposure to *n*/260 butyric acid, the time of exposure to 32° required for optimal development was found to range from 5 to 7 minutes; one minute's exposure to *n*/260 butyric acid appears thus physiologically equivalent to warming at 32° for the same or a somewhat longer period. After-treat-

TABLE XV.

n/260 BUTYRIC ACID WITH AFTER-TREATMENT WITH SEA-WATER AT 34°.

August 27. The eggs from one starfish were used. The eggs were few in number, but the majority showed normal behavior. They were exposed to *n*/260 butyric acid for one minute and then returned to sea-water. Later (within 20 minutes) portions were exposed to hypertonic sea-water, cyanide, and warm sea-water as indicated.

After-treatment.	Results.
1. None (control)	Typical membrane-formation, followed by breakdown of almost all eggs. One blastula found.
2. Hypertonic sea water for 30 m.	35–45 per cent. of the eggs form larvæ.
3. *n*/1000 KCN for 30m.	*Ca.* 50 per cent. of all eggs form larvæ.
4. 34° for 1 min.	Only a few eggs form larvæ: < 1 per cent.
5. 34° for 2 min.	Marked improvement: 20–30 per cent. form larvæ.
6. 34° for 3 min.	Larvæ are fewer than in Exp. 5: *ca.* 20 per cent.
7. 34° for 4 min.	Few eggs form larvæ: < 1 per cent.
8. 34° for 5 min.	Most eggs fail to divide; none form larvæ.

Warming at 34° without previous membrane-formation: Eggs were exposed to 34° in the usual manner for 2, 3, 4, 5, 6, and 7 minutes. The best development resulted from the 2- and 3-minute exposures, with respectively 25–35 per cent. and 35–40 per cent. of eggs forming larvæ; with the 5-minute exposure only 5 per cent. formed larvæ.

ment with sea-water at 31° and at 34° was also tried; the results were the same except that the after-exposure required at 34° was only a half to a third as long as at 32°, and at 31° about twice as long. The following series at 34°(Table XV.) is typical.

These results show that the effective duration of after-exposure at 34° is about one third of what it is at 32°; at 31° the best results were gained with after-exposures of 8 to 10 minutes. The temperature-coefficient of the physiological change resulting from the after-warming treatment is thus evidently of the same order as in the case of simple warming without previous membrane-formation. This of course is not surprising, since undoubtedly the same process is concerned in activation by heat whether this is preceded by another treatment or not.

It is thus plainly a matter of indifference, as regards the effect

TABLE XVI.

BOTH MEMBRANE-FORMATION AND AFTER-TREATMENT BY *n*/260 BUTYRIC ACID

September 6. The eggs from one starfish were used; these were few in number, but almost all (*ca.* 90 per cent.) showed normal maturation, and in the sperm-fertilized control almost all formed larvæ. The eggs were exposed for one minute to *n*/260 butyric acid and returned to sea-water; part were left in sea-water as control; the remainder were again placed, 18 minutes later, in *n*/260 butyric acid, from which portions were returned to normal sea-water at the intervals indicated. These eggs developed as follows:

After-treatment.	Results (Condition of Eggs after 4 Hours and Proportion forming Larvæ).
1. None (control)	All show typical membrane-formation followed by irregular change of form and breakdown in nearly all eggs. Only one larva seen.
2. *N*/260 Butyric acid: 2 m.	A few eggs are cleaved. *Ca.* 10–15 per cent. form larvæ.
3. Butyric acid: 4 m.	Cleavages more numerous and more regular than in Exp. 2. *Ca.* 40–50 per cent. of eggs form larvæ.
4. Butyric acid: 6 m.	Cleavages still more numerous: Most eggs form larvæ (70–80 per cent.).
5. Butyric acid: 8 m.	Like Exp. 4, but fewer eggs form larvæ (50–60 per cent.).
6. Butyric acid: 10 m.	Cleavages are fewer and slower. 25–35 per cent. of eggs form larvæ.
7. Butyric acid: 12 m.	Cleavages are still fewer. *Ca.* 10 per cent. of eggs form larvæ.
8. Butyric acid: 15 m.	Practically none are cleaved. No larvæ.

produced by this form of after-treatment, whether the membrane-formation is induced by heat or by fatty acid; in either case warming for a few minutes completes the process of activation and enables the eggs to develop favorably. Precisely the same effect is gained by after-exposing eggs, in which membranes have been formed by either method, to weak solutions of fatty acid for a brief period; the effects of such treatment are in all respects similar to those of after-warming. This is illustrated by the following experiment (Table XVI.).

It is clear that in the time-relations of its action as well as in its other characteristics, this form of after-treatment resembles closely that with warm sea-water. It is also possible to treat the eggs first with warm sea-water and then after-treat with butyric acid solution; precisely the same results follow as in the experiment just described. This is illustrated by the following series (Table XVII.).

TABLE XVII.

BRIEF EXPOSURE TO 32° WITH AFTER-TREATMENT BY $n/260$ BUTYRIC ACID.

September 7. The eggs from one starfish were used; eggs were few but apparently normal, over 90 per cent. showing normal maturation, and sperm-fertilization resulting in a large proportion of larvæ. The eggs were exposed to sea-water at 32° for 3 minutes, then returned to sea-water at normal temperature, and 16 minutes later placed in $n/260$ butyric acid solution, from which they were again returned to sea-water after the times indicated.

After-treatment.	Results.
1. None (32° for 3 m. alone)	No development; only a small proportion form membranes.
2. $N/260$ butyric acid: 2m.	All form membranes but few are cleaved after three hours. *Ca.* 5 per cent. form larvæ.
3. Butyric acid: 4 m.	A large proportion (50–60 per cent.) are cleaved after three hours. More than 50 per cent. form larvæ.
4. Butyric acid: 6 m.	Most eggs are cleaved after three hours. 70–80 per cent. form larvæ.
5. Butyric acid: 8 m.	In contrast to Exp. 4, few eggs are cleaved after three hours, and only 1–2 per cent. form larvæ.
6. Butyric acid: 10 m.	No eggs cleave within three hours. None form larvæ.

The favorable effect of this after-treatment is evident. It will be noted that the three minutes' exposure to 32° was insufficient for membrane-formation in most eggs; but the effect of this preliminary treatment is seen in the fact that an after-exposure of only 4 minutes was sufficient to induce development to larval stages in more than half of the eggs. After-exposure to butyric acid solution has the same favorable effect when the preliminary warming is sufficient to form membranes in all eggs; in a second similar series on September 12 the eggs were exposed for 4 minutes to 32° and all mature eggs thus treated formed membranes; without any after-treatment almost none (less than 1 per cent. formed larvæ, but with an after-treatment of 4 to 8 minutes with *n*/260 butyric acid favorable development took place in a large proportion of eggs.

General Discussion and Conclusion.

The interchangeability of the treatments with warm sea-water and butyric acid solution indicates that both agents produce their effect by inducing the same kind of change in the egg-system. This change is evidently of a "releasing" kind, and initiates the sequence of developmental processes; these, once started, continue automatically to their conclusion. Probably their most distinctive peculiarity is the highly specific character of the chemical transformations that take place. From the food contained as reserves in the egg, or taken in from the surroundings, the developing germ builds up the specific compounds which form the structural basis of the organism; this synthetic process, in the case of the chief structure-making compounds, the proteins, undoubtedly starts—as in the constructive metabolism of the adult animal—with the amino-acids, which are recombined in the specific manner predetermined by the chemical organization of the germ. Bodies of the most highly specific and individualized physical and chemical properties are thus built up and laid down in definite positions as development proceeds. Their properties and their spacial disposition determine at any time the character of the transformation undergone by the building material which is being incorporated. According to this conception it is the chemical specificity of these substances that determines the specific character of development in the more evident or morphological sense,[1] *i. e.*, why the egg gives rise to an individual of the same species; and we must therefore be prepared to find among the earliest chemical changes associated with development, interactions of a specific kind—*i. e.*, specific in the sense in which the interaction of antigen and antibody is specific—between complex substances already present in the egg. There is now definite experimental evidence that such reactions do in fact constitute an essential part of the fertilization-process.[1] Specific substances which apparently unite in fertilization (since after fertilization they are no longer demonstrable) are present in the unfertilized mature egg; one of these ("fertilizin") may be largely removed from the egg by washing, and when this is done fertilization is prevented. If such specific unions are essential to fertilization, we must conclude that the specific substances concerned in this process are in some way kept from interaction in the resting mature egg, and that the activating agent removes this hindrance to interaction. The question which I wish briefly to discuss in this section relates to the nature of this inhibiting condition, and the manner in which the activating agent effects its removal.

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[1] Reichert's work on the crystal-forms of haemoglobin and other complex compounds from different species of animals and plants constitutes perhaps the

The nature of the effects following exposure of unfertilized eggs to temperatures of 30°–35° indicates clearly that activation does not depend on simple acceleration of some chemical process, *e. g.*, oxidation, which is already proceeding in the egg, since in this case the temperature-coefficient of the activation-process would presumably show the usual value of $Q_{10} = 2–3$. It is also evident that heat-coagulation is not concerned, since these temperatures are too low, and the readiness with which the activation process can be arrested by cooling and renewed by a second warming shows that its basis is some effect which is completely reversible by change of temperature. These characteristics, high temperature-coefficient and reversibility with change of temperature, are however shared by the typical melting and gelation (sol-gel transformation) exhibited by solutions of gela-

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best evidence of this. The morphological characters of crystals and crystal-aggregates varies with their chemically specific ("species-specific") character in a definite and constant manner. It is fair to assume that the influence of these compounds in determining organic structure depends largely on the kind of aggregates they form. *Cf.* Reichert: *Science*, 1914, N. S., Vol. 40, page 649.

[1] *Cf.* F. R. Lillie, *Journal of Experimental Zoology*, 1914, Vol. 16, p. 523.

tine, agar, soaps, lipoids and other hydrophilous colloids. The relations of temperature to this process show in fact a close resemblance to those described above for the activation-process. One striking peculiarity of melting and gelation is that both processes take place gradually; when (*e. g.*) a gelatine sol is brought below the gelation-temperature and the conditions are then kept constant, the actual solidification takes place only after the lapse of a considerable period of time. The time required to reach the gelation-stage decreases rapidly as temperature is lowered; thus Levites found that a gelatine sol kept undisturbed at 26° took 26 hours to gelatinize, at 25° only 11 hours.[1] The first observable change in the solution is an increase in viscosity; this continues until the system sets; the setting represents the end-stage of the whole process, whose course can thus be traced by successive viscosity-determinations. Gelation is thus equivalent to a progressive increase in viscosity to a final stage at which the ordinary fluid mobility is lost.[2] It is found that above a certain temperature the viscosity of the hydrosol undergoes no change with time; but if the temperature is lowered a critical point is eventually reached below which the viscosity undergoes steady increase (at a rate dependent on temperature, presence of salts, reaction) until gelation occurs. The rate of this increase in viscosity (*i. e.*, of the gelation-process), $\Delta\eta/\Delta t$, shows a high temperature-coefficient. With a 1 per cent. gelatine solution Schroeder[3] obtained the following values for the viscosity at 21°, 24.8°, and 31° at different intervals after bringing the warm gelatine solution to the temperature of observation:

Interval.	Viscosity Observed at		
	21°.	24.8°.	31°.
5 min.	1.83	1.65	1.41
10 min.	2.10	1.69	1.41
15 min.	2.45	1.74	1.42
30 min.	4.13	1.8	1.42
60 min.	13.76	1.9	1.42

Thus while at 31° the viscosity undergoes no change with time, at the lower temperatures there is a steady increase. If we take comparatively short time intervals, *e. g.*, 10 minutes, we find that the value of $\Delta\eta/\Delta t$ at 21° $\left(\frac{2.45 - 1.83}{10} = 0.062\right)$ is about seven times greater than at 24.8° $\left(\frac{1.74 - 1.65}{10} = 0.009\right)$. In other words, a difference of about 4° increases the average rate of the gelation-process from six to seven times. What is true of the gelation-process is also true of the inverse degelation or melting process, whose rate increases at a similarly rapid rate with rise of temperature above the critical maximum at which the system remains permanently in the gel state.[1]

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[1] Levites, *Kolloid-Zeitschrift*, 1907, Vol. 2, p. 211.

[2] *Cf.* Schroeder, *Zeitschrift für physikalische Chemie*, 1903, Vol. 45, p. 75; Levites: *loc. cit.*, p. 209; Freundlich, "Kapillarchemie," 1909, pp. 416 ff.

[3] Schroeder, *loc. cit.*, p. 88.

In starfish eggs the rate of the activation-process, at temperatures between 30° and 36°, shows a similar proportionate increase with a given rise of temperature, as will be seen by reference to Table X.; *i. e.*, the temperature-coefficients of the two processes, gel-sol transformation, and activation of the egg under the influence of high temperatures, are similar in their order of dimensions; thus a rise of 4° shortens the time of exposure necessary to cause membrane-formation or development by six to ten times. On the assumption that some specific chemical interaction is the essential change in the initiation of development, such a result indicates that the rate of this interaction is dependent, in the case of parthenogenesis by warming, on the rate of some process involving either degelation or decrease in the viscosity of some portion of the colloidal system of the egg. This is as much as can be inferred on the basis of these facts alone. If we also take into account the other methods by which membrane-formation and activation can be induced, we are led to the further inference that this colloidal change affects chiefly if not exclusively the surface-layer (cortical zone or plasma-membrane) of the egg. Thus typical membrane-formation can be induced by brief treatment with pure isotonic solutions of neutral salts.[2]

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[1] On account of the hysteresis of the gelatine system, the melting temperature is typically several degrees higher than the solidification-temperature; it is also higher after the gel has stood some time than immediately after solidification. *Cf.* Pauli (Pascheles): *Archiv f. d. ges. Physiologie*, 1898, Vol. 71, p. 336.

[2] R. S. Lillie: *American Journal of Physiology*, 1910, Vol. 26, p. 106. The fact

whose action is certainly superficial, as well as by substances like fatty acids, weak bases, and lipoid-solvents, which readily penetrate the plasma-membrane. Those neutral salts of sodium and potassium which are the most effective in inducing membrane-formation, iodides and thiocyanates, are also the most effective in lowering the melting points of protein gels and in promoting water-absorption by such gels.[1] Such facts suggest that the salts act in a way similar to that of high temperatures, *i. e.*, by furthering degelation of surface-structures or absorption of water in the surface-layer of the egg. The effect of such an increase in water-content would be to increase the general permeability of this region, since according to the experiments of Bechhold, Ruhland, and others[2] the permeability of gels to diffusing substances, especially to colloids, is a direct function of their water-content.

High temperature, according to this interpretation, acts like other parthenogenetic agents, by increasing the permeability of the surface-layer,—this effect resulting directly from some change in the nature of a degelation or decrease in the viscosity of the colloidal system in this region. Apparently the immediate effect of this change is to allow a chemical interaction to take place between substances which in the normal resting state of the surface-layer are kept apart. The general fact that identical physiological effects may be produced by lipoid-solvents, and by substances which appear to alter the membrane by interacting chemically with its constituents,[3] indicates that the integrity of the plasma-membrane as a semi-permeable partition is the essential factor in preserving the resting condition of the egg.[4]

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that this action can be prevented by anesthetics confirms the view that it depends on an increase in the permeability of the plasma-membrane: *cf.* my recent paper in the *Journal of Experimental Zoology*, 1914, Vol. 16, p. 591.

[1] *Cf.* Pauli (Pascheles): *Archiv f. d. ges. Physiologie*, 1898, Vol. 71, p. 333; Levites: *loc. cit.*; Pauli and Rona, *Beiträge zur chemischen Physiologie u. Pathologie*, 1902, Vol. 2, p. 4.

[2] Bechhold u. Ziegler, *Zeitschr. f. physik. Chem.*, 1906, Vol. 56, p. 105; also, "die Kolloide in Biologie u. Medizin," 1912, p. 48. Ruhland: *Biochemische Zeitschrift*, 1913, Vol. 54, p. 59; Freundlich, *Kapillarchemie*, pp. 515 *seq.*

[3] When membrane-forming substances act by combining chemically with egg-constituents, it is to be expected that the rate of action will vary with temperature in accordance with the chemical temperature-coefficient. *Cf.* the experiments of Loeb and Hagedoorn, "Artificial Parthenogenesis and Fertilization," page 146.

[4] *Cf.* my paper, *Amer. Journ. Physiol.*, 1911, Vol. 27, p. 289.

Hence it is a matter of secondary importance in what manner this semi-permeability is temporarily destroyed, provided that the condition of increased permeability lasts long enough—not too long—and is not associated with irreversible changes making recovery impossible. It is presumably during this stage of increased permeability that the above specific interaction takes place; this process requires time, and its rate will be a function of the rate at which the two interacting substances can come together; this second rate will be a function of the viscosity or gelation-state of the protoplasmic system at the site of interaction,—hence its dependence on temperature, as seen above. When this critical interaction has taken place, there follows at once the characteristic change of physiological activity normally resulting from fertilization; membrane-formation and the other events preparatory to cell-division occur and the developmental process proper is initiated. How far development proceeds, however, depends on the degree of completion of the primary specific reaction; hence for complete activation the exposure to the membrane-forming condition must have a certain minimal duration, and in case the preliminary exposure is insufficient some after-treatment may be necessary to complete the process. This after-treatment may be of the same kind as the preliminary membrane-forming treatment, or it may be of entirely different kind—*e. g.*, hypertonic sea-water, cyanide, an anaesthetic, etc. But there seems to be no need of assuming that its direct physiological effect is qualitatively different from that of the membrane-forming agent.[1] It merely renews and brings to completion a process already initiated by the first treatment.

Comparative study of the conditions of both normal and artificial activation ought to yield data from which by elimination the essential factors common to the two processes may be determined. Judging from the data available at present, the most general common feature appears to be the initial increase in permeability.[1] It is not yet clear, however, how this change can be the means of initiating the specific interaction assumed. The substances which interact are assumed to be present in advance in the egg; how is their interaction prevented by the existence of a semipermeable surface layer? The connection between change of permeability and activation is probably indirect; and the analogy to stimulus and response in the general stimulation-process of irritable tissues still seems the best adapted to throw light on this question.[2] In stimulation an electrical depolarization of the plasma-membranes of the irritable elements is apparently the critical event; in some way this change enables the characteristic response of the irritable system to take place. Similarly in the initiation of development in the unfertilized egg. The agents which induce membrane-formation in eggs have typically a depolarizing action on irritable cells like muscle-cells—*i. e.*, cause a negative electrical variation.[3] Such a change appears to result whenever surface-permeability is increased; and it seems therefore probable that this depolarization, as such, is what enables the union of specific substances—the first step in activation—to take place. We may assume that one of the interacting substances is situated immediately beneath the electrically polarized surface-film of the egg, that it is a negative colloid, and that its tendency to unite with some amboceptor-like body also present in this region is compensated by the electrostatic attraction between it and the layer of positive ions immediately external to the egg-surface. Depolarization would then permit interaction to take place.[1] Such a conception, while in a sense diagrammatic, helps at least to explain how a non-specific agency, provided it only alters sufficiently the boundary-layer of the egg, can be the means of initiating such a highly specific process as development.

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[1] The above experiments are a sufficient justification of this contention. But they do not explain why, for instance, after-treatment with cyanide, which by itself does not induce membrane-formation in starfish eggs (*cf. Journal of Experimental Zoology*, 1913, Vol. 15, p. 38), is so effective. Clearly the condition of the egg after membrane-formation is altered so that the activation-process may then be influenced by agents which previously had no effect upon it (as cyanide, alcohols, or hypertonic sea-water in brief exposure). Sensitization to these agents seems to be involved in the process of membrane-formation, but the basis of this effect can not be defined at present. There is, however, no necessary inconsistency between these facts and the conception that the activation-process is essentially unitary in character in the above sense. The case of hypertonic sea-water offers certain special problems, which are partly discussed below.

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[1] *Cf.* my paper just referred to. In a recent paper Gray confirms McClendon in finding a temporary increase in the electrical conductivity of sea-urchin eggs immediately after sperm-fertilization. *Cf.* Gray, *Journ. Mar. Biol. Ass.*, 1913, Vol. 10, p. 50; McClendon, *American Journ. Physiol.*, 1910, Vol. 27, p. 240.

[2] I have discussed this analogy in more detail in the paper above cited (footnote 2, p. 296); also in the *Journal of Experimental Zoology*, 1913, Vol. 15, p. 23.

[3] For the action of cytolytic substances in producing local negative variation, *cf.* Straub, *Archiv f. exp. Path. u. Pharm.*, 1902, Vol. 48, p. 1; *Zeitschr. f. Biol.*, 1912, Vol. 58, p. 251; Henze: *Arch. f. d. ges. Physiol.*, 1902, Vol. 92, p. 451; Hermanns: *Zeitschr. f. Biologie*, 1912, Vol. 58, p. 261; Allcock; *Proc. Roy. Soc.*, B, 1906, Vol. 77, p. 267; *Journal of Physiology*, 1906, Vol. 33, p. xxviii; Evans, *Zeitschr. f. Biol.*, 1913, Vol. 59, p. 397.

The discussion of this question can hardly be considered complete without some reference to the case of hypertonic sea-water. As Loeb has shown, exposure to this agent forms a supplementary treatment which is remarkably favorable with some eggs, especially sea-urchin eggs. This treatment seems to occupy a special position among the parthenogenetic agents. It may either precede or follow the membrane-forming treatment,[2] and in some way it puts the egg into a condition which is favorable to subsequent development; this action seems quite independent of the nature of the membrane-forming or activating agent, and so far it has received no satisfactory explanation. Loeb has shown that a purely physical abstraction of water is not the only factor concerned; a chemical factor, apparently involving oxidation, is essential; free oxygen must be present during the treatment, and the effective times of exposure vary at different temperatures according to the chemical temperature-coefficient.[3] Some hypothesis as to its mode of action seems required; and I suggest the following, which is consistent with the foregoing point of view, and has not, to my knowledge, yet been put forward.

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[1] The inorganic analogy would be, *e. g.*, the interaction between solution and metal at the surface of the plate in a battery when the circuit is closed. While the battery is at rest (with open circuit), interaction between (*e. g.*) sulphate ions and zinc is prevented by the polarization at the surface of the zinc plate. The tendency to this ionic interaction is compensated by the polarization, the zinc ions being held back by the negatively charged plate. Similarly, *mutatis mutandis*, with the reactions at the cell-surface, or other surfaces (adsorption-surfaces) within the cell. The facts of stimulation afford in general strong evidence that the chemical processes in the living cell are largely dependent on changes in the electrical polarization of the limiting membranes. *Cf.* my paper in the *Journal of Biological Chemistry*, 1913, Vol. 15, p. 237. Also, for a more general discussion of this question, the article entitled "The Physico-chemical Conditions of Stimulation," in the *Popular Science Monthly*, 1914, p. 579.

[2] *Cf.* Loeb, "Artificial Parthenogenesis and Fertilization," Chapter 11; *Archiv für Entwicklungsmechanik*, 1914, Vol. 38, p. 409.

[3] "Artificial Parthenogenesis and Fertilization," Chapter 11.

It is to be assumed that the activation-process—as the earliest step in development, an essentially constructive process—involves syntheses of some kind. Now the intracellular as well as other organic syntheses consist as a rule, in the union of two or more molecules, with loss of water, to form larger molecules,—as in the formation of fats from glycerol and acids, of starch and glycogen from sugar, of polypeptides and proteins from amino-acids, etc. In order to account for the readiness with which these condensations occur in cells, it seems necessary to assume that the protoplasm is the seat of energetic dehydrations, probably in certain localized situations (possibly at membranes or other adsorption-surfaces). The artificial enzymatic synthesis of triolein from glycerol and oleic acid has been found to take place readily only when water is removed as completely as possible from the reacting mixture.[1] Hence the synthesis of fats by enzyme action in cells is intelligible only on the assumption that in the region of their formation there is energetic abstraction of water or dehydrolysis. Certain biological facts indicate that partial removal of water from cells is favorable to syntheses of the above kind. According to Overton, plasmolysis of plant-cells furthers the formation of starch in chloroplasts.[2] Butkewitsch also finds that the formation of starch in the amylase-rich cortex of certain plants (*Sophora*, *Robinia*) is promoted by placing in strong sugar-solutions (10–20 per cent. dextrose and saccharose).[3] The observations of Pavy and Bywaters and of Rubner on the formation of glycogen by yeast cells in strong sugar solutions constitute probably a further instance of the same phenomenon.[4] In general loss of water will be favorable to—since it will supplement—the action of any dehydrating mechanism; and it is possible that in the sea-urchin egg after membrane-formation the intracellular dehydration-processes are by themselves not quite energetic enough to effect the syntheses necessary for initiating development, but become so when supplemented by the action of the hypertonic sea-water; *i. e.*, this agent has the effect of reducing the concentration of water at the *locus* of the reactions sufficiently to enable syntheses to take place which otherwise are impossible under the conditions. It is significant that cell-division is started in the sea-urchin egg by simple membrane-formation, but fails to continue,—just as if there were some failure in the supply of the necessary constructive materials; partial abstraction of water rectifies this condition. Since oxygen is necessary to this corrective process, we may assume that the syntheses belong in part to the class designated by Schmiedeberg[1] as oxidative syntheses.

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[1] *Cf.* the papers of Pottevin: *Comptes rendus de l'Académie*, 1903, Vol. 136, p. 1152, and 1904, Vol. 138, p. 378; Taylor, *Journal of Biological Chemistry*, 1906, Vol. 2, p. 87; Hamsik, *Zeitschr. f. physiol. Chemie*, 1909, Vol. 59, p. 1; Armstrong and Gosney, *Proceedings Roy. Soc.*, Ser. B, 1914, Vol. 88, p. 176.

[2] Overton, *Vierteljahrsschrift d. naturf. Ges. in Zürich*, 1899, Vol. 44, pp. 131–2.

[3] Butkewitsch, *Biochem. Zeitschr.*, 1908, Vol. 10, p. 314; *cf.* pp. 336 seq.

[4] Pavy and Bywaters, *Journal of Physiology*, 1907, Vol. 36, p. 149; Rubner, *Archiv für Physiologie*, Suppl. 1912, p. 252, and *ibid.*, Vol. for 1913, p. 244.

Pavy and Bywaters found that in pure dextrose solutions the deposition of glycogen in yeast cells increased rapidly with increase in the concentration of dextrose up to an optimum. In 2 per cent. solutions there was little effect; in 4 per cent., 8 per cent., and 16 per cent. solutions there was a rapid progressive increase in the quantity of glycogen laid down in the cells to a maximum of over 13 per cent.

From this general point of view the action of hypertonic sea-water becomes in a measure theoretically intelligible and ceases to be merely a detached empirical fact. Certain avenues of experimental approach to the problem are also suggested.

SUMMARY.

§ 1. The effects following exposure of maturing unfertilized starfish eggs to high temperatures (29–36°) vary in a constant manner with the times of exposure as follows. Below a certain minimal duration of exposure to any given temperature (*e. g.*, 32°), no visible change is produced in the egg; slightly longer exposures induce the formation of typical fertilization-mem-

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(as compared with about 5 per cent. under normal conditions); in more concentrated solutions there was a decline. They also found that too long exposure to a favorable solution (10 per cent.) was unfavorable; thus yeast incubated in 10 per cent. dextrose for 2½ hours showed an increase in glycogen-content from 4.84 per cent. to 11.66 per cent.; four hours later there was a decline to 9.33 per cent. These facts show a suggestive parallel with the effects of hypertonic sea-water on sea-urchin eggs; here also there is no effect until a certain minimal osmotic pressure is reached; with further increase in osmotic pressure there is a rapid increase in favorability up to an optimum; still further increase is unfavorable. Also for a favorable concentration there is at any temperature a definite optimum time of exposure.

[1] *Cf.* Schmiedeberg, *Archiv f. exper. Pathologie u. Pharmakologie*, 1893, Vol. 31, p. 281.

branes, but the eggs fail to cleave and soon break down without development; in order to induce favorable development an exposure of three to four times the minimum for membrane-formation is required (*e. g.*, 7–8 minutes at 32°); more prolonged exposures are again followed by failure to develop.

2. Between 29° and 38° the times of exposure required to produce these effects decrease very rapidly with rise of temperature; on the average a rise of 1° approximately halves the exposure required for a given physiological effect (such as membrane-formation, or complete activation, or heat-inactivation). The activation-process thus exhibits a characteristically high temperature-coefficient ($Q_{10} = 200–400$).

3. The effects of exposure to weak butyric acid solution ($n/260$) vary with time of exposure in a similar manner,—brief exposure causing membrane-formation followed by breakdown, longer exposures causing cleavage and development to larval stages, and still longer exposures causing cytolysis without development.

4. The inference is that the same process is initiated in the egg by exposure to warm sea-water as by fatty acid solution. This process must proceed to a certain stage in order that activation may be complete; if arrested too soon (brief exposure) only partial activation (membrane-formation followed by breakdown) results.

5. Eggs in which membranes are formed by minimal exposure to warm sea-water or $n/260$ butyric acid, followed by return to sea-water, may be made to develop favorably by a second treatment with either warm sea-water or fatty acid solution, as well as by after-treatment with cyanide-containing or hypertonic sea-water. A favorable after-treatment may thus be of the same kind as the membrane-forming treatment.

6. The temperature-coefficient of activation by high temperatures is of the same order as that of the melting of gels or the decrease in the viscosity of gelatine solutions. The above high temperatures thus probably act by producing degelation-effects in the surface layer of the egg; increase of permeability, with consequent depolarization, is the result of this change.

7. A new hypothesis of the mode of action of hypertonic sea-water is put forward.

DIVISION RATE IN CILIATE PROTOZOA AS INFLUENCED BY THYROID CONSTITUENTS.[1]

ROBERT A. BUDINGTON AND HELEN F. HARVEY.

INTRODUCTION.

In the very numerous studies which have been made to ascertain the effect of thyroid tissues and extracts on growth and differentiation, the material employed, whether used as a food for large organisms or as a component of a medium in which to breed smaller forms, has been taken in a very large proportion of instances, if not always, from some mammal, *e. g.*, cow, horse, or sheep. This has been the case even though the animal under observation may have been a mammal, a bird, an amphibian, or a protozoan.

Assuming that the doctrine of evolution is a fairly probable hypothesis it is only a natural if not necessary corollary that each of the several organs involved, as well as the organism as a whole, has experienced its own successive changes, its own evolutionary modifications. Variations, "continuous" and "discontinuous," have occurred in internal as well as in external organs, and these variations have involved the physiological value of the organs concerned, as well as their anatomy; so that, of glandular tissues, for example, the composition of the output has undergone phylogenetic changes, so to speak, during the process of descent of one phylum from another. It is *a priori* improbable, of course, that the chemical composition, and consequent stimulating potency, of the thyroid secretion is the same throughout the entire vertebrate phylum.

Apparently the earliest experimentation along the line with which this paper deals was carried out by Nowikoff ('08), who found that one effect of putting sheep thyroid into the medium in which *Paramœcium* was living was to cause it to divide more rapidly than normally.

Recently, Shumway ('14) has published a paper in which he verifies Nowikoff's contentions. Both these investigators, however, employed mammalian thyroid; and, since our results agree with theirs, the question for mammalian thyroid, at least, seems fairly well settled.

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[1] From the Department of Zoology, Oberlin College.

Nowikoff's work suggested to us the query whether or not his results could be taken as widely significant.[1] Our purpose has been, therefore, to add to the known facts along this line by ascertaining the influence of glands taken from each of the five main subdivisions of vertebrated animals, so far as they or substances derived from them, affect certain protozoa; and, using division-rate as an index, to thus get a line on the comparative physiology of this gland.

MATERIALS AND METHODS.

Perfectly fresh thyroid glands were taken from the fresh-water sucker (*Catostomus teres*), the frog (*Rana pipiens*), the turtle (*Cistudo carolina*), the chick (*Gallus domesticus*), and the cat (*Felis domestica*), dissected as cleanly as possible from surrounding tissues, and then dried by moderate heat; each was then ground to powder in a mortar, and the material then kept in vials till used. In the instance of the mammalian gland, fatty tissue was present in such amount that this was dissolved away by repeated washing in ether to bring the gland to such condition that it could be finely pulverized. In supplying thyroid material to protozoa in this form, we endeavored to avoid any alteration in its character such as might result in the making of glycerin or alcoholic extracts. This seems a point which should be rather carefully guarded.

The forms employed for experimentation were *Stylonichia* and *Paramœcium*. To familiarize ourselves with a method of handling such organisms, and also for the purpose of securing individuals whose ancestry would be known to us, we first carried isolated "wild" forms through a considerable number of generations (in the case of *Stylonichia*, seventy-four); we employed depression slides, kept in a moist chamber, each slide carrying four drops of bacterial hay infusion made up in the manner of that used by Woodruff ('05) in much of his work. The "wild" specimens were taken from ordinary laboratory cultures, but the particular individuals used in any given experiment were taken from the pedigreed lines descended from a single parent. The protoplasm of the line treated with thyroid was identical with that of the control carried beside it.

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[1] Our work was completed before Shumway's article appeared.

The procedure in any experiment was this: two protozoa of common parentage were isolated, each in four drops of the same culture medium. To one of the slides was added a minute mass of pulverized gland, which would thus influence the protozoan either as a food, or as a factor in the environing medium so far as this acted as a solvent.[1] The actual amount of each pulverized gland thus used was small, and a like amount of each was determined as closely as possible by careful subdivision of a slightly larger mass on a clean paper surface. To weigh out the powder would give no more equal amounts, inasmuch as the glands are so invaded by vascular and connective tissue that any moiety taken might easily contain more or less of other than glandular material. A slight amount of fresh hay infusion was added to each slide each day, and the experiment continued six days or more. The results given in this account are limited to those obtained during the first six days only; to keep track of the offspring of even a single protozoan longer than this is extremely difficult, as many know. The effect of each different gland was tested by three trials.

Circumstances were such that it was not always convenient or possible to run experiments with all five different thyroids at one time, so a control was carried along beside the gland-fed individual in each case. This assured that the same conditions of every sort attended both experimental and control lines, no matter when the observations were made. If any circumstance favored or interfered with either, the same was true for the other.

EXPERIMENTAL FINDINGS.

The following tables show the exact results, so far as number of individuals resulting from division of the original one goes, this rate of cell division being the only index of thyroid effect at present ready for presentation. The ciliate used in the first series of experiments was *Stylonichia;* in the second and third series we used *Paramœcium.* While the evidence is too limited to permit any rigid conclusion of the kind, the data at hand seem to indicate that *Paramœcium* is rather more susceptible to thyroid ingredients than is *Stylonichia.*

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[1] Shumway states in his recent paper, *loc. cit.*, that more or less of the material thus offered *Paramœcium* is actually ingested and digested.

Results from Use of Fish Thyroid.

	First Experiment, No. of Individuals.		Second Experiment, No. of Individuals.		Third Experiment, No. of Individuals.	
	Control.	Thyroid-fed.	Control.	Thyroid-fed.	Control.	Thyroid-fed.
1st day	1	1	1	1	1	1
2d day	4	2	1	2	1	1
3d day	6	6	3	2	6	6
4th day	15	15	5	7	14	16
5th day	24	37	7	50	18	23
6th day	43	49	15	90	52	73

Results from Use of Amphibian Thyroid.

	First Experiment, No. of Individuals.		Second Experiment, No. of Individuals.		Third Experiment, No. of Individuals.	
	Control.	Thyroid-fed.	Control.	Thyroid-fed.	Control.	Thyroid-fed.
1st day	1	1	1	1	1	1
2d day	1	1	1	2	1	2
3d day	6	5	2	4	8	18
4th day	7	11	8	9	16	76
5th day	7	12	10	30	25	127
6th day	12	30	12	61	36	243

Results from Use of Reptilian Thyroid.

	First Experiment, No. of Individuals.		Second Experiment, No. of Individuals.		Third Experiment, No. of Individuals.	
	Control.	Thyroid-fed.	Control.	Thyroid-fed.	Control.	Thyroid-fed
1st day	1	1	1	1	1	1
2d day	4	4	4	5	1	2
3d day	8	10	8	23	3	7
4th day	8	10	8	47	15	42
5th day	8	21	14	148	23	80
6th day	8	38	15	362	50	276

Results from Use of Avian Thyroid.

	First Experiment, No. of Individuals.		Second Experiment, No. of Individuals.		Third Experiment, No. of Individuals.	
	Control.	Thyroid-fed.	Control.	Thyroid-fed.	Control.	Thyroid-fed.
1st day	1	1	1	1	1	1
2d day	1	4	1	2	2	1
3d day	2	9	3	7	8	16
4th day	2	10	4	45	23	32
5th day	4	15	15	63	59	141
6th day	5	24	35	243	91	399

RESULTS FROM USE OF MAMMALIAN THYROID.

	First Experiment, No. of Individuals.		Second Experiment, No. of Individuals.		Third Experiment, No. of Individuals.	
	Control.	Thyroid-fed.	Control.	Thyroid-fed.	Control.	Thyroid-fed.
1st day	1	1	1	1	1	1
2d day	13	15	4	8	2	2
3d day	16	18	6	18	9	18
4th day	18	20	10	48	34	59
5th day	30	132	14	60	56	129
6th day	53	306	29	253	90	487

For convenience in seeing at a glance the relation which held between the experimental lines and the controls during the use of any particular kind of thyroid, the data for the three experiments with each thyroid have been averaged, the controls for the same averaged, and the results placed in curve form.[1] These follow:

FISH.

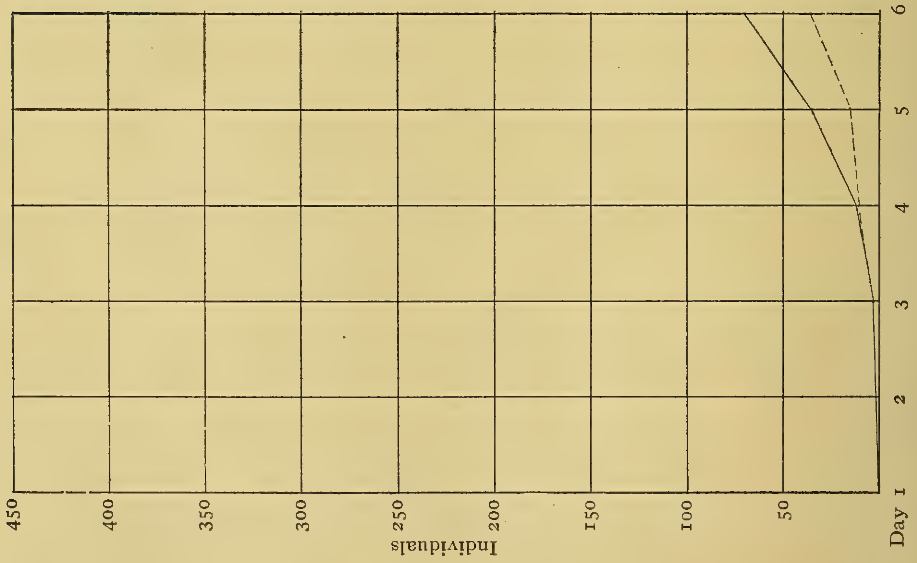

CHART I. Showing number of individuals at end of each day; the unbroken line = those treated with fish thyroid; broken line = the control.

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[1] An erroneous conclusion is rather easily drawn from these charts, for at first glance it appears that the potential of the gland increases by steady gradation from the fish up to the mammal. When figured as percentage increases of the experimental over the control lines, it is found that such is not the case. Data pertaining to this relation are now being collected.

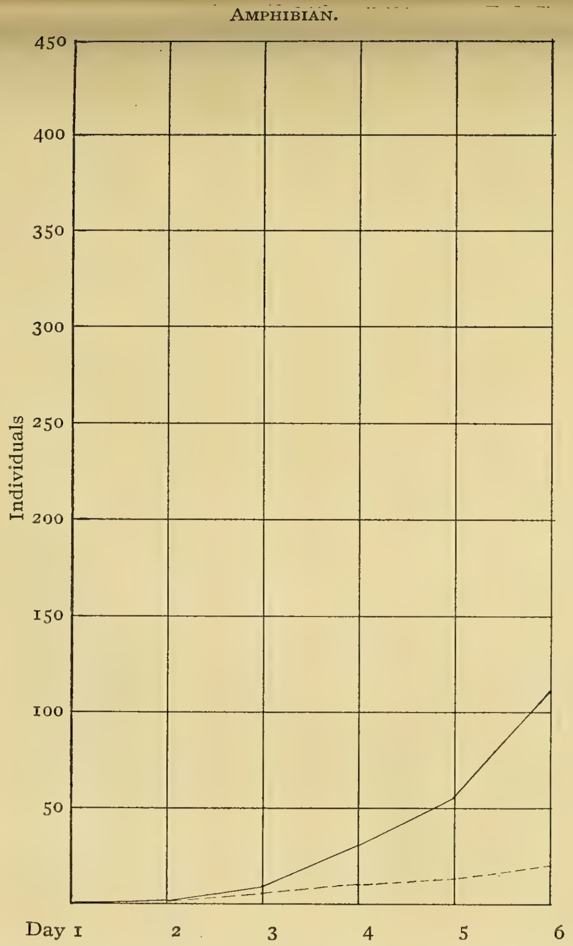

CHART 2. Plotted in same manner as Chart 1, but showing result when one of two sister individuals was given amphibian thyroid.

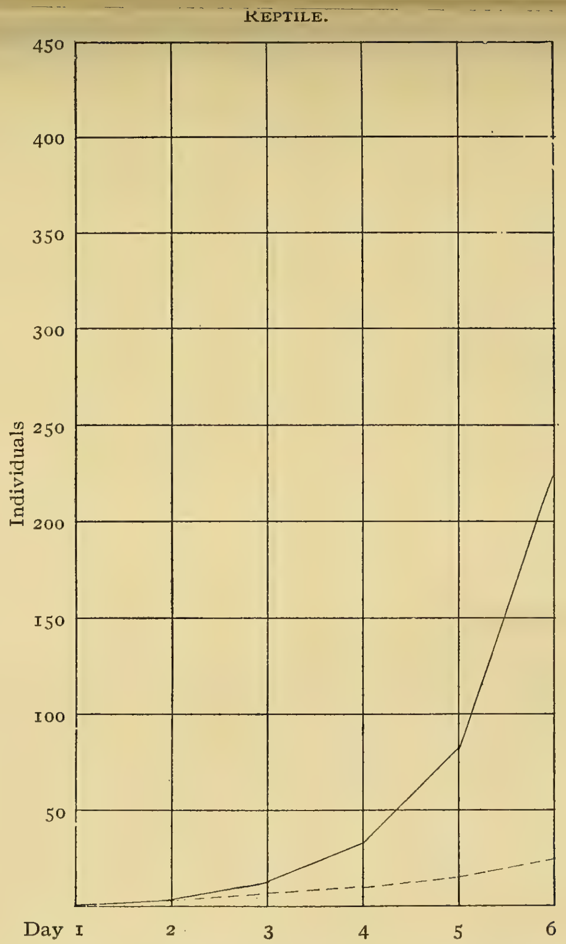

CHART 3. Plotted in same manner as Chart 1, but showing result secured with reptilian thyroid.

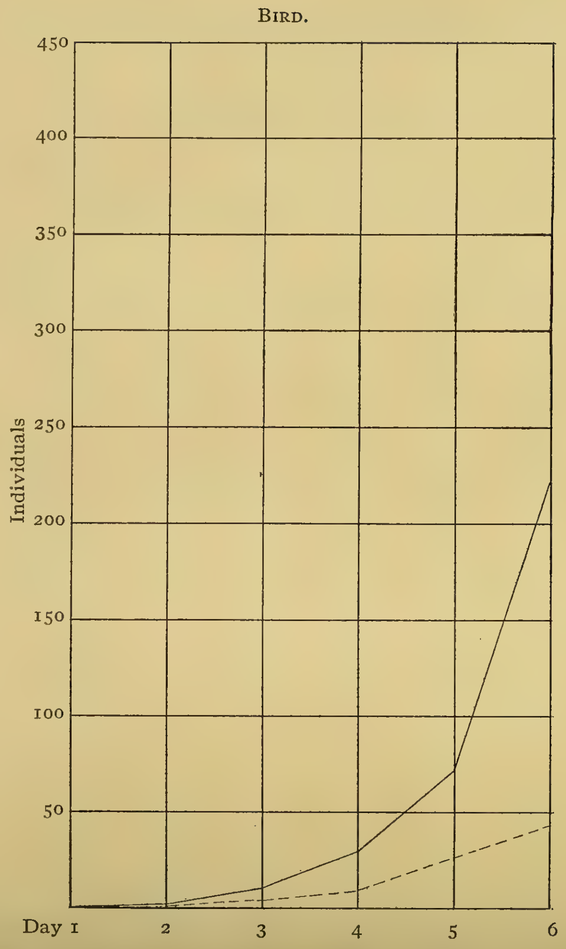

Chart 4. Plotted in same manner as Chart 1, but showing result secured with avian thyroid.

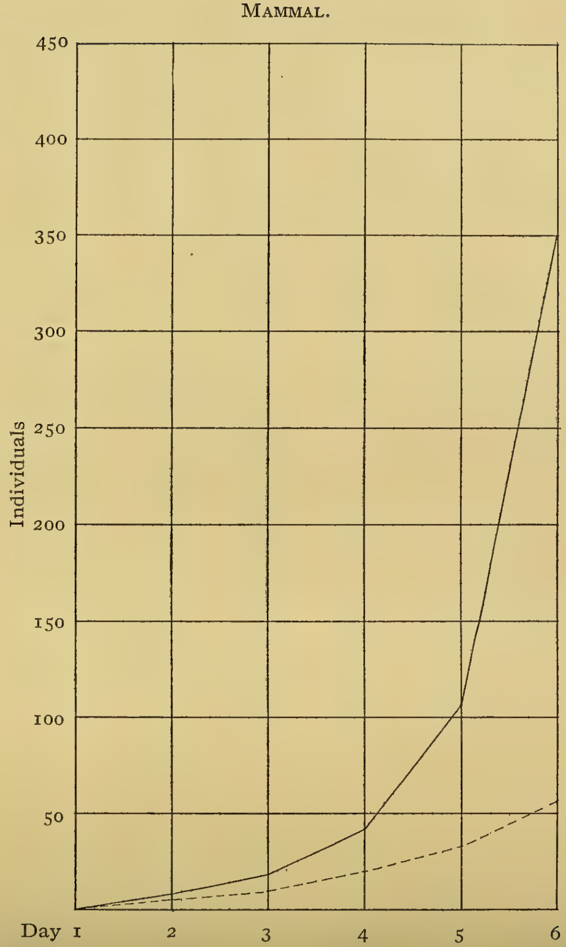

Chart 5. Plotted in same manner as Chart 1, but showing result secured with mammalian thyroid.

A curve plotted from the averages of all five of the different experimental lines and drawn beside a curve portraying the averages of the control lines for the same periods, each figured day by day for the six days, represents, in a manner, a generalized curve of the effect of vertebrate thyroid constituents on protozoa, as based on our data. This curve takes the following form:

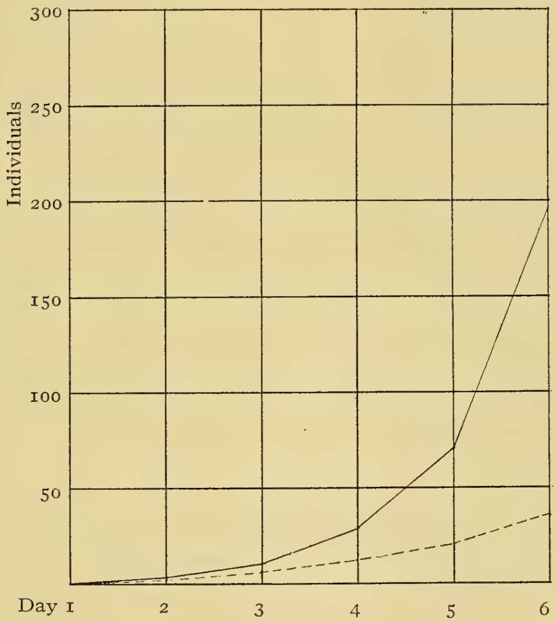

CHART 6. A curve plotted from the averages of Charts 1, 2, 3, 4 and 5, and thus representing a composite of results obtained from use of thyroids from each of the five main classes of vertebrates.

DISCUSSION.

The number of papers which have hitherto been published along this line seems to be limited to those already mentioned; so that conclusions have to be drawn from a comparatively meager literature, and to gather largely around the investigator's own experience. There is essential agreement between the findings of Nowikoff, Shumway, and ourselves as to the constant effect of thyroid ingredients in increasing the division rate of protozoa beyond the normal, at least so far as *Paramœcium* is concerned.

The work of others, notably Gudernatsch ('12, '13), on the feeding of amphibian embryos, seems to indicate that the effect there observed is mainly one of acceleration of differentiation of tissues in the growing organism; at least this is the interpretation given their findings. West ('14) has verified certain features of Gudernatsch's results. A similar betrayal of specialization in function would, of course, not be possible within the limits of a unicellular organism. It seems entirely probable, however, that intra-cellular modifications of the *Paramœcium* protoplasm does accompany its feeding upon and living in a medium which, among other things, brings it hurriedly to its most crucial experience, self-division. The fact that rapid fission of thyroid-fed *Paramœcia* is accompanied by their increased activity and transparency, and by smaller size,[1] indicates that very important internal modifications doubtless occur. Careful study of protozoa exposed to exigencies of this sort should be made.

If cell division in protozoa is to be compared with anything in the life history of metazoa, it should certainly be considered beside the early development of the metazoan egg. If the egg has already advanced to the proportions of an embryo or larva, and the precocious differentiation of tissues and organs in such is under consideration, the question arises: Is this differentiation at all explained in the same terms as is protozoan cell division, or does it involve the same basic factors? It seems to us that this query may very possibly be answered in the affirmative, for the reason that the sprouting out of legs from the tadpole and establishment of other organs characteristic of the adult, is surely not due to mere unusual division of labor among the young cells generally acting as little more than unit components of the infant tadpole body; but that these latter have been provoked (by thyroid ingredients?) to *abnormally rapid division*, probably with accompanying abnormally small size, and that *entirely normal differentiation* has set in among cells which have been derived by the abnormally early (rapid) multiplication of their ancestors.

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[1] Shumway mentions these alterations to occur in thryoid-fed *Paramœcia*, and we have found such to be practically always observable.

We would suggest, therefore, that there may be, at bottom, not any great difference between what shows itself in Gudernatsch's work as differentiation, and the result which shows itself as cell division in an animal where differentiation, so far as it exists, can assert itself only intra-cellularly, and thus in a very obscure manner.

It is certainly entirely unnecessary to dwell upon the obvious fact that the more nearly adult a metazoan animal is, the more difficult it becomes to even suggest parallelisms which may exist between it and unicellular organisms; so that, to discuss the numerous physiological effects which have been obtained from feeding thyroid tissues to various vertebrata, or from grafting and transplantation experiments, or to examine the studies of conditions in higher types provoked by pathological thyroid growth and disease, is quite beyond the scope, if not impossible in connection with the subject, of this paper.

Summary.

The conclusion to which the foregoing experimental results point is that thyroid ingredients, no matter from what class of vertebrates the gland be taken, produce essentially the same result when given to ciliate protozoa (*Paramœcium* and *Stylonichia*) as a food or as a factor in the medium in which they live, viz., increased division rate.

The tissue which has hitherto been used in experimental work along this line has, we believe, always been taken from mammals. We think it safe to say that, no matter how far apart taxonomically, or how distantly related phylogenetically the "higher" and "lower" members of the vertebrate phylum may be, certain physiological qualities in the thyroid glands are constant and similar in all.

Sufficient difference exists between the potential of the thyroid secretion of one vertebrate class and that of another, so that, if studies of the normal value of this gland are being made, glands from the same class, if not from the same genus and species of animal as the one under observation, may well be employed.

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AN EXPERIMENTAL STUDY OF THE MOVEMENTS OF HERRING AND OTHER MARINE FISHES.[1]

VICTOR E. SHELFORD AND EDWIN B. POWERS.

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I. INTRODUCTION.

The general problem of increasing the supply of any species of fish or any other aquatic food animal, or of maintaining such species against extensive catch and against pollution of waters with sewage and the waste products of manufacturies, is very complex. The older methods of study are as important now as ever. The study of the food of an animal, its relation to its natural enemies, and its breeding habits still must receive their proper share of attention. In addition to these we now know that attention must be given to the chemical condition of the water, its effect on the movements and migrations and general health of the animals. Likewise it is especially important to study the physical and chemical conditions in which the animals breed and to look especially into the matter of the *preservation of the natural breeding grounds.* It is well known that one of the reasons for the depletion of the white-fishes in Lake Michigan is the destruction of their breeding grounds by the addition of sewage, saw-dust and other refuse to the water, which has settled on the breeding grounds and rendered them uninhabitable by the lowering of the oxygen content and covering the surface with materials which bury and tend to smother the eggs during development. The number of individuals of a species is never any greater than the breeding grounds can support. Finley ('13) has shown that the number of prairie chickens in certain counties of Illinois is directly proportional to the area of breeding grounds. Likewise the senior author (Shelford, '11) has shown that in a series of ponds at the head of Lake Michigan, food fishes are absent where their food is *greatest in quantity* because the breeding conditions are absent, due to the covering of the bottom with the decaying food of fishes. It is especially noteworthy that the food of the youngest fishes is especially abundant in ponds where the best food fishes cannot breed. This is not due to the failure of young fishes to destroy the small crustacea, because the same principle holds for ponds in which there are as many crustacea-eating fishes in stages suitable for *food* fishes as in stages suitable for only non-food fishes.

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[1] Contribution from the Puget Sound Marine Station.

The economic justification for the study of the movements of fishes is two fold. First experimental studies are concerned with the question of the conditions which the fishes select or reject when presented with two or three kinds of water to which they have free access under experimental conditions. Their importance in this connection is based upon the fact that so long as we are concerned with conditions which the fishes habitually encounter in nature, the selections or rejections represent in a general way the physiological character of the fishes and as a rule conditions which fishes reject are detrimental if continued for a long time. Thus, as we shall see later, fishes turn away from water containing hydrogen sulfide and we will show further that they die very quickly when exposed to only a small excess of this gas in the sea water. Here then the fish is so constituted that its behavior and safety are intimately linked. Of course there are exceptions to this rule and it does not hold when we are concerned with changes in conditions which are not commonly encountered in nature. Thus we learn something of the conditions that are probably deleterious to the animals without either killing them or breeding them continuously under the modified conditions. The second justification lies in the fact that we can learn by such experiments the effect of advancing civilization and industry upon the presence or absence of a species in any locality. The movements of the fishes must be known as well as the cause therefor, before we can intelligently approach the question of capturing them in quantities.

II. Material and Methods.

1. *Stock of Fishes.*

The material used in these experiments was chiefly the fry of the herring (*Clupea pallasii* Cuvier) 6 cm. (2½ in.) common in Puget sound. The fry were caught on July 2 and were kept in a float car anchored in a good tide and until July 22 practically none of the fish died except during the first few days when those probably injured in catching were the chief victims. A few soles (*Lepidopsetta bilineata* Ayres) secured on July 4 at Fisherman's bay, Lopez Island, were kept in the same car. A few young hump-back salmon (*Oncorhynchus gorbuscha* Wal.) 7 cm. (2¾ in.) long were secured at sea, through the courtesy of Dr. E. Victor Smith on June 30, at Turn Island and were not used after July 8 as they did not appear to be in normal condition after that date. A single Cottid (*Oligocottus maculosus* Girard) was used in killing experiments. The soles and herring appeared to be in essentially as good condition at the end of the period of work as at the beginning.

2. *The Water Supply of the Station.*

Experiments were run in which both fresh and salt water were used. Thus it is necessary to consider the character of both. The fresh water in use during the summer of 1914 was supplied by the village of Friday Harbor and came from deep wells. Owing to the rocky character of the ground in the vicinity, it was impracticable to bury the pipes and the temperature varied greatly with the weather, night, day, etc. The highest temperature noted was 24° C. The water contained an excess of gas which escaped in a cloud of bubbles when it was withdrawn from the tap. This was neither oxygen nor carbon dioxide and gave no odor which points to the conclusion that it was nitrogen. The water was distinctly alkaline to phenolphthalein, free carbon dioxide being wanting. The half bound carbon dioxide was 24.2 c.c. per liter and the fixed 28.6 c.c. The oxygen was less than 0.5 c.c. per liter (for methods see Birge and Juday, '11, pp. 13–21). Such water is unsuitable for biological purposes and was used in these experiments only after aëration by running it slowly over an inclined board ten inches wide and four feet long. After this aëration the oxygen content was, at 13° C., 4.9 c.c. per liter and the excess of other gas was removed, but the water still remained alkaline.

The sea water supplied at the station building was pumped from a depth of about four feet below mean tide. It was retained in a wooden tank, being pumped twice per day, in the evening and in the morning. Upon standing in the tank the temperature rose from 11° to nearly 15° on warm days.

The oxygen was determined by the Winkler method. In no case was the sea water from the tank or from the bay from which it was pumped, saturated with oxygen even in samples collected at the surface. The only surface collection made that showed saturation according to the tables of Fox (see Murray and Hjort, '12, p. 254) was from the strong tide rips off Point Caution at 5:30 P.M. Collections from the bottom of sandy shores among *Ulva* were super-saturated.

Chlorine was determined by titrating with silver nitrate. It usually amounted to about 16.93 grams per liter. It was usually a little higher in water from the tank than in water collected from the sea. The determination of carbon dioxide was made by the method in common use in fresh water. The sea water was titrated with $\frac{1}{20}$ normal solution of sodium carbonate, with phenolphthalein as an indicator. The water was usually acid in reaction indicating about 1.7 c.c. per liter of free carbon dioxide. The half bound and bound carbon dioxide as indicated by the method used by Birge usually amounted to 25.3 c.c. per liter each. There was considerable uniformity in the results of such titrations and while the method is not especially accurate the lack of oxygen common in the water would indicate an excess of free carbon dioxide over that commonly reported for sea water. The correctness of these figures is further suggested by the slight alkalinity of the water taken from the vicinity of green algæ and containing an excess of oxygen.

Hydrogen sulfide is very commonly present in sea water when decomposition is taking place. This was determined by titration with iodine which was the only method we were equipped to employ. It is never present in any quantity in freely circulating waters. The highest records are for collections made near the bottom under *Ulva*, where the odor is often quite distinct. On account of the probable presence of other substances which may absorb iodine the determinations may be slightly too large (Birge and Juday, '11).

TABLE I.

THE DISSOLVED GASES OF THE SEA WATER ABOUT FRIDAY HARBOR, WASHINGTON. DATA IN C.C. PER LITER.

Date.	Place.	Hour.	Tide.	Collected.	CO_2.	O_2.	H_2S.	Temp.
	Point Caution	5:30 P.M.	Low, in	Surface		5.6		
7/23	N. E. Brown's Id.	10:10 A.M.	Low, out	Surface	1.76	4.9	.187	11.6
7/25	Do.	11:10 A.M.	Low, out	Surface	1.64	4.6	.237	10.7
7/25	Do.	7:15 P.M.	High,	Surface	1.91	4.6	.268	10.5
7/26	S. Brown's Id.	10:45 A.M.	Low, in	8″ under *Ulva*	0.00	9.2	.536	16.5
7/23	Do.	12:00 M.	Low, in	Do.	0.00	10.8	.536	13.2
7/25	Do.	12:00 M.	Low, in	18″ Do.	0.00	—	.339	13.2
7/25	Station dock	11:10 A.M.	Low, in	Surface	1.86	5.2	.149	11.6
7/25	Do.	12:45 P.M.	Low, in	Surface	3.10	4.8	.205	
7/26	Do.	9:30 A.M.	High, out	Do.	1.81	4.2	.295	10.6
7/26	Tap-pumped at	6:30 A.M.	Med. low	4′ deep	1.76	4.7	.223	

It will be noted from a study of the table that the water from Point Caution where the tide has full sweep is the only water saturated with oxygen at the surface. In other places the sea water at the surface is about 1 c.c. less than the amount given by Fox (see Murray and Hjort, '12, p. 254). Aërating the sea water increased the oxygen. The water from the tank did not seem to have been modified by standing for sixteen hours or more. On the whole there must be much decomposition in Puget Sound waters. There was no constant difference between the water from outside and inside the side of the island which encloses Friday Harbor. The CO_2 is a little higher except at low tide in the sample taken near the *Ulva;* the oxygen remains about the same. The hydrogen sulfide does not average appreciably higher.

The explanation for the alkaline character of the water under the *Ulva* is that the plants take up the CO_2 and give off oxygen and thus remove the excess which occurs in other localities.

The absorption of oxygen in connection with the development of the hydrogen sulfide probably prevents any very great excess of CO_2 from accumulating (Lederer '12).

III. The Resistance of Fishes to Contamination and Decomposition Products.

It was not possible to try the resistance of the fishes (Wells, '13) to the effect of the lack of oxygen either separately or in combination because no means of removing it was at hand. It was possible only to add gases to the water. Hydrogen sulfide and carbon dioxide were used.

1. *Herring* (*Clupea pallasii Cuvier*).

Hydrogen sulfide is extremely poisonous to the fishes (Weigelt, '03). In the first attempted gradient experiments where the water at one end contained only a little of the gas the fishes turned on their backs in two or three minutes when the one inflow was showing 8.3 c.c. per liter and the other was pure sea water. This happened in spite of the fact that more than half of the time was spent in the end with least H_2S. The experiments were performed in the manner described by Wells. When placed in a solution of 7.6 c.c. per l. the herring gasped *after 1 minute and 45 seconds*, turned over after 5 minutes, and were apparently all dead in 6 minutes. In carbon dioxide of about 20 c.c. per l. the herring showed evidence of loss of equilibrium after three minutes. Some of them sank to the bottom after 12 minutes. After 39 minutes to 62 minutes herrings turned on their sides on the bottom, resting in this position for a time and then swimming more nearly normal for a time again. One died after 102 minutes, the others after 159 minutes' exposure. The oxygen was about 5.5 c.c. per l. and varied directly with the amount of tank CO_2 used, indicating that the carbon dioxide contained much oxygen.

When carbon dioxide and hydrogen sulfide were used together the carbon dioxide was about 30 c.c. per l. and the hydrogen sulfide 2.9 c.c. per l. The amounts were controlled with some difficulty and thus the experiments are not alike in the matter of concentration. Herring were much stimulated at the beginning.

After 1 minute and 30 seconds there was a general loss of correlation of movements. At the end of four minutes all the herring were dead. Thus we note that the combination of hydrogen sulfide and carbon dioxide is exceedingly deadly. In alkaline partly aërated fresh water herring showed loss of equilibrium in from 10 to 14 minutes. They nearly succumbed and then recovered a few times, the first one dying after 30 minutes and all being dead in 44 minutes.

2. *Soles* (*Lepidopsetta bilineata Ayres*).

In the hydrogen sulfide (7.6 c.c. per l.) the soles showed some signs of loss of equilibrium at the end of one minute. In 5 minutes they were on their backs. After 13 minutes they had revived again. They were nearly dead after 16 minutes and all dead at the end of 24 minutes.

In the carbon dioxide (20 c.c. per l.), after 45 minutes one sole gasped, which was the first sign of any disturbance and one turned on its back after 54 minutes. For three hours this was repeated at intervals and each gasping time was followed by recovery.

In the combined carbon dioxide (30 c.c. per l.) and hydrogen sulfide (2.9 c.c. per l.) the soles lost equilibrium after 2 minutes and 30 seconds. In 11 minutes they were barely alive and in 13 minutes were dead. In fresh water the soles showed stimulation at the end of 3 minutes. They died in from 48 minutes to one hour.

3. *Cottid* (*Oligocottus maculosus Girard*).

One fish of this species was added from curiosity but the results were sufficiently surprising to record. In the hydrogen sulfide the cottid seemed unaffected until the end of 6 minutes, after the herring were all dead. It breathed heavily after 16 minutes. The fish was alive at the end of three hours when it was returned to running salt water, and allowed to recover, after which it was used in the carbon dioxide experiment, with similar results. In the combined carbon dioxide and hydrogen sulfide it was not visibly affected and in fresh water there was no evidence of any disturbance. These fishes were seined from the sandy bottom among the *Ulva*, coming in with numbers of the small soles.

4. *Summary.*

We note that on the whole the presence of a quantity of carbon dioxide in the water affected the fishes less than a smaller amount of hydrogen sulfide. The combination of hydrogen sulfide and carbon dioxide was most rapidly fatal. Since decomposition yields CO_2 and consumes oxygen and is accompanied by the production of hydrogen sulfide which is also accompanied by the consumption of oxygen, it is reasonable to suppose that on a bottom from which vegetation is absent and decomposition actively takes place a fatal combination of lack of oxygen, and presence of hydrogen sulfide and probably carbon dioxide can develop quickly.

Considering the fishes tested we note that the herrings were most sensitive. They were sharply marked off from the bottom species which are resistant to a marked degree. This resistance is in a very general way associated with the habitat preference of the species. Still the marked resistance of the small cottid is not quite explicable on this or any other basis.

The importance of factors which kill fishes is greatest in the early stages for two reasons. First the small size of the eggs and embryos makes the ratio between volume and surface smallest and thus any substance in solution will reach all parts of the organism at a more rapid rate. Secondly the inability of the eggs and embryos to move about makes them the easy victims of any adverse conditions that may occur. The eggs of the herring are deposited on the bottom. Nelson mentions rocks only (Marsh and Cobb, '10, p. 46) and rocks are usually swept fairly clear of organic matter and the water well aërated down to the depth of one fathom where the fishes breed. If this means that sandy bottoms of bays are avoided it probably means the avoidance, during the breeding, of water high in hydrogen sulfide (see table) which would be fatal to the eggs and small herring fry to a greater degree than to those studied, which were 6 cm. long. Sensitiveness to hydrogen sulfide is a matter of much importance from the standpoint of the suitability of a given arm of the sea for herring and the influence upon fishes of contamination of the shores with refuse from the land.

Carbon dioxide is not high in such shallow water on account of the presence of so many green plants. Carbon dioxide is probably more important in connection with movements of the fishes than in the matter of restricting their breeding places.

IV. Reactions of Fishes to Chemical Conditions in Sea-Water.

1. *Conditions and Methods of Study.*

The experiments were performed in a gradient tank. Water of two kinds was used in the experiments. One kind was allowed to flow into one end at a definite rate and another kind into the other end at the same rate. It flowed out at the middle at the top and at the bottom so that the two kinds of water met at the center. The outflow at the center did not of course prevent the mixing of the two kinds of water and thus the middle section, equal to one half or one third of the tank was a gradient between the two kinds of water. The tank used in these experiments was 122.3 cm. by 15 cm. by 13 cm. deep. The front wall was of plate glass and a plate glass top was used at times. Water was allowed to flow in at both ends at the same rate (usually 600 c.c. per minute) through tees the cross bars of which contained a number of small holes. The cross bars of the tees were at the center of the ends of the tank behind screens. The drain openings were located at the center near the top and in the bottom. The outer openings of the drain tubes were at the level of the water in the tank. The water flowed in at the ends and drifted toward the center and flowed out through the drains. We found no evidence that fishes react to the slight current thus produced. Since each half of the tank held about 9 liters, it required 15 minutes to fill it or to replace all the water in one of the halves. The tank was enclosed under a black hood. Two candles (electric lights being wanting) were fixed in the rear and above the center of the two halves, *i. e.*, above a point midway between the screen partition and the center drain. The light was 15–20 cm. above the surface of the water which was 13 cm. deep. The room was darkened during the experiments which were observed through openings in the hood above the lights or through the glass side late at night. Fishes do not usually note objects separated from them by a light.

Water differing as little as possible from that in which the fishes usually live was used for control readings. Controls were observed and the conditions in the two ends of these were the same either because the water introduced at the two ends was alike or because no water was run into either end (standing water).

In the controls the fishes usually swam from end to end in a rather symmetrical fashion, and thus by comparing these movements with those occurring when the fishes encountered differences in water, we are able to determine the reactions of the fishes to the differences. Various kinds of water were used at one end as follows: (1) water with varying amounts of carbon dioxide added; (2) water with oxygen added; (3) water with hydrogen sulfide added; (4) fresh water.

When the difference between the solutes at the two ends of the tank was not great we found by chemical tests that the central portion of the tank was a gradient between the characteristic waters introduced at the two ends. Usually the end thirds were essentially like the inflowing water. When the difference in concentration was great the region of the gradient was proportionally longer and the ends with the inflowing concentrations correspondingly shorter. When the difference in concentration was very great the entire tank was gradient. For an experiment a fish was placed in a dish containing enough water to barely cover it and set above the tank. When all was in readiness the fish was emptied into the center of the tank. Marks on the sides divided the tank into thirds. The fish nearly always swam back and forth, apparently exploring the tank. The movements of the fish were recorded graphically as shown in Chart I. For this purpose sheets of ruled paper were used. Four vertical double rulings corresponded to the thirds and two ends of the tank. Distance from right to left was taken to represent the length of the tank, vertical distance to represent time and the graphs drawn to scale. The width of the tank was ignored. The graphs on the following pages are copies of the originals.

Before or after the experiment, the headings of the sheets were filled with data regarding the kind, size, and previous history of the fish, the conditions in the tank, concentration of the solutes and other significant data. The fish was observed continuously for twenty or more minutes.

In order to maintain a constant flow the water was introduced into the tank by means of siphons from cans on the top of the hood with a 74 cm. head. Connected with one of the two cans was an inclined plank trough 420 cm. by 25 cm. for the purpose of aërating water before it entered the can if so desired.

By the method just described it is possible to obtain unusually accurate data on the factors influencing the movements of fishes. According to Marsh and Cobb ('07) a great difficulty in the herring fishery is the erratic movements of the fish. Schools may visit a bay for three or four years, in succession, and then without any apparent reason, avoid it for a season or two or altogether. Bertham ('97) noted a possible relation between the abundance of these fishes and weather and suggests that climatic cause may have more to do with the failure of some branches of the fisheries than is generally believed. He attributed the failure of the fisheries of Cape Benton to the occurrence of severe east and northeast storms during the running season. It is not clear what the effect of such storms may be, but they chiefly affect the dissolved content of the water. Johnstone, '08, page 246, says that it is now nearly certain that the shoaling migrations of the herring of Europe are to be associated with the salinity and temperature of the sea. It is evident from these experiments that acidity and alkalinity are more important than salinity and the solution of the problem will come from a careful study of the reactions of fishes along with a similar study of hydrographic conditions.

2. *Reactions to Temperature.*

These fishes are remarkably sensitive to differences in temperature. We obtained good reactions with a difference of 0.6° C. in the length of the tank. Fair reactions were obtained with differences of 0.5° C. and since the fishes often turned around near the center it appears that they recognized a difference of 0.2° C. In graph 1, Chart I., we show the reaction of fish in a gradient of 0.6° C. (compare with graph 2—control). The fish was taken from sea water at 10.9° and the experiment performed at 12.8° and 13.2°. It will be noted that the fish showed a preference for the higher temperature. Eleven experiments were performed with herring and in seven cases the fishes showed a preference for the warmer water and in three cases for the colder. One did not show any marked preference. The differences were too slight to be of great significance in determining whether the fishes move into warmer or colder water but show a great sensitiveness. Thus temperature may play an important rôle in the movements of fishes.

It will be noted by reference to the graph, that the fish moved into the colder water several times as if trying out the entire tank and then turned back periodically from the colder end. In the control where there was no flow or difference in temperature the fish turned back from both ends at times but by chance as shown by other controls, turned a little more often from the end corresponding to the cold end of the experiment due perhaps to difference.

3. *Hydrogen Sulfide.*

The animals turned back sharply from all concentrations not great enough to cause intoxication as shown in graph 3, Chart I. (compare with control graph 4). In this experiment the hydrogen sulfide was only 4.5 c.c. per l. and the fishes avoided it sharply and after trying out the tank turned about at a point where the concentration could not be more than one tenth of that at the treated water end or about equal to that under the *Ulva* on the south side of Brown's Island (p. 319).

This experiment is typical of several and the fishes are thus seen to be able to orient with reference to an increase in the solute and to turn back from it very sharply.

The control (graph 4) to this experiment is symmetrical, there being turning from both ends in equal number. It shows the reaction of the fishes when no stimuli are encountered in the tank.

4. *Reactions to Salinity, Acidity and Alkalinity.*

As noted above, the fresh water of the laboratory was from deep wells and not good for biological work. It was alkaline, containing no free carbon dioxide, 24.2 c.c. per l. half bound and

28.6 c.c. per l. of bound carbon dioxide. There was a deficiency of oxygen and what was present was probably due to leaky pipes. It was only 0.5 c.c. per liter. It contained a large excess of some odorless gas which escaped in bubbles and was probably nitrogen. This water was aërated by running it over a board 420 cm. long, into the siphon bucket. This reduced the gas in excess to air saturation and raised the oxygen to 4.8 c.c. per liter.

In the experimental tank the difference between the density of the fresh and salt water was so great that the fresh extended nearly to the opposite end at the top with very little mixing and the salt water occupied a corresponding place on the bottom. Thus there was a sharp gradient from top to bottom, but a very imperfect one from end to end. To avoid this siphons were inserted which withdrew water from each side near the bottom at a point one third the length from the salt end and from near the top at the same distance from the fresh end. This was found not to remedy the difficulty sufficiently and so a screen incline which extended from bottom at the salt end to the height of 8.5 cm. at the fresh end. Above this was another screen which was 8.5 cm. at the salt end, and which ran up to the surface of the water at the fresh end. This enclosed the fish in an inclined cage 8.5 cm. deep at the salt water end and 5.0 cm. deep at the fresh end. The fish moved back and forth in this at a distance of about 4 cm. from the lower screen. The gradient of salinity between the acid sea water and the alkaline fresh water was essentially as perfect as shown in the accompanying Fig. 1. By consulting this figure it will be seen that the oxygen content was essentially the same throughout. The salinity corresponded to 10.561 grams of chlorine in the salt water end to 6.45 grams in the fresh water end. The acidity to phenolphthalein reached

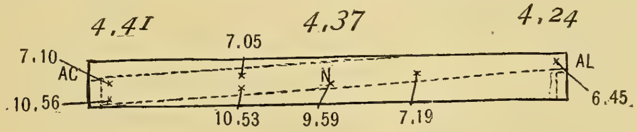

FIG. 1.

FIG. 1. Showing the distribution of salinity in terms of grams of chlorine per l. in Roman and oxygen content in c.c. per l. in italics; Al, alkaline; N, neutral; Ac, acid.

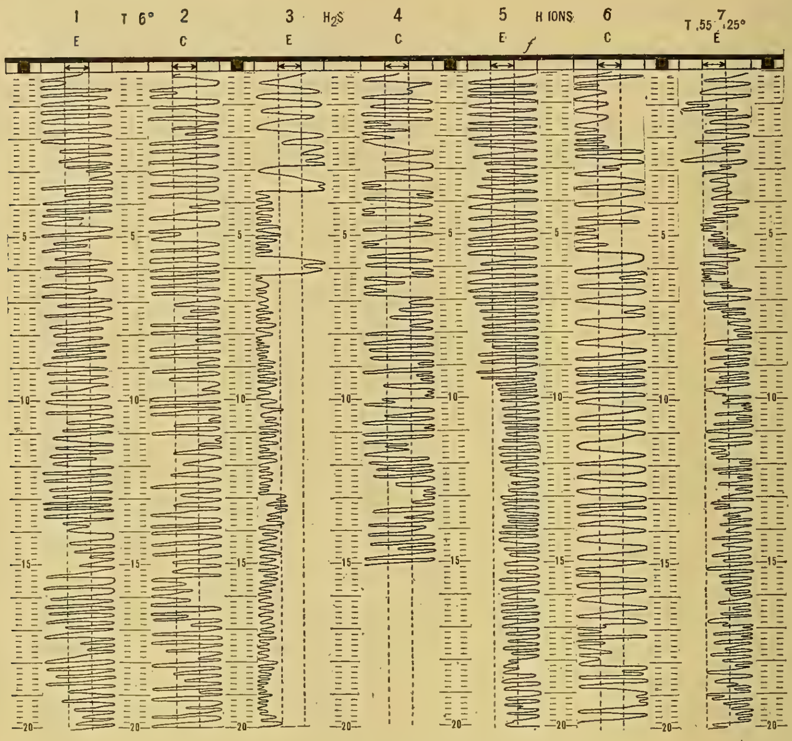

CHART I.

E, experiment; *C*, control; *f*, fresh water, *T*, and figures following show temperature difference.

Graph 1 shows the reaction to a difference of 0.6 of a degree the lower avoided temperature being on the left. Graph 2 shows the movement of the fishes in the tank when there is no difference in temperature (see also graph 6 and other graphs marked *C*).

Graph 3 shows the avoidance of hydrogen sulfide introduced at the right. After a few trials the avoidance became very sharp. Graph 4 is the control, *i. e.*, with no difference between the ends.

Graph 5 shows the reaction of a fish to fresh water introduced at the right showing the avoidance of the acid salt water and selection of the alkaline fresh water with the incline described on p. 327 in position. Temperature the same at the two ends. Graph 6 is the control of the same.

Graph 7 shows the selection of lower temperature with the incline screen cage in position; difference in beginning .55° C., at end .25° C. A difference in temperature occurred in some of the incline experiments but lower at the salt end. The graph shows that the fishes would have selected the salt water end where the temperature was a little lower if they had been reacting to temperature.

almost to the center while the central region was essentially neutral. Consulting graph 5, Chart I., we note that the fish moved the entire length of the tank for two minutes and then began to turn back before the highest salinity was reached. After a few such turnings it went the entire length of the tank for a short period with one exception. Between the 7th and 14th minutes the excursions into the salt water were gradually shortened. In other words after a few brief entrances into the salt water the fish gradually shortened its invasions of the salt water until it was turning rather regularly just on the alkaline side of neutrality, which continued to the end of the observation. It will also be noted that the fish turned back twice from the fresh water end, which is significant because in other cases the fishes selected this region. This was true in four other experiments with the incline and in six out of eleven performed without the incline. It appears that the herring select either brackish or slightly alkaline water. The control, graph 6, is symmetrical.

In some of the experiments performed with the incline there was a slight difference in temperature between the two ends, the fresh water being a little higher. To check this source of error, the experiment was performed with the incline but with the difference in temperature reversed, and the fishes selected the opposite end of the tank, showing that this was not only a reaction to solutes but that the solutes inhibited any reaction to temperature that might otherwise have taken place (graph 7). In the temperature experiments the fishes selected the higher temperature when the stock was fresh and the lower temperature near the close of the work showing that the fishes had undergone some slight physiological change during their stay in the float-tank.

The tendency to come to rest in the region on the alkaline side of neutrality was very clearly shown in all the experiments except one. The salmon oriented with their heads toward the fresh water end, drifting very slowly back, probably floating in a current and then swimming up again to the same point. This was very striking and constituted an unmistakable difference between the experiment and control. Chart II., graph 13 and 14, show such an experiment and control. The swimming up occurred notably in the 13th and 18th minutes.

To determine whether or not this peculiarity is a reaction to salinity or alkalinity, the experiment with herring was repeated and carbon dioxide to which the fish are negative (graphs 8 and 9) run in the fresh water, to neutralize the alkalinity. At the beginning of the experiment shown in Chart II., graphs 10 and 11, the carbon dioxide content of the fresh water was 26.5 c.c.

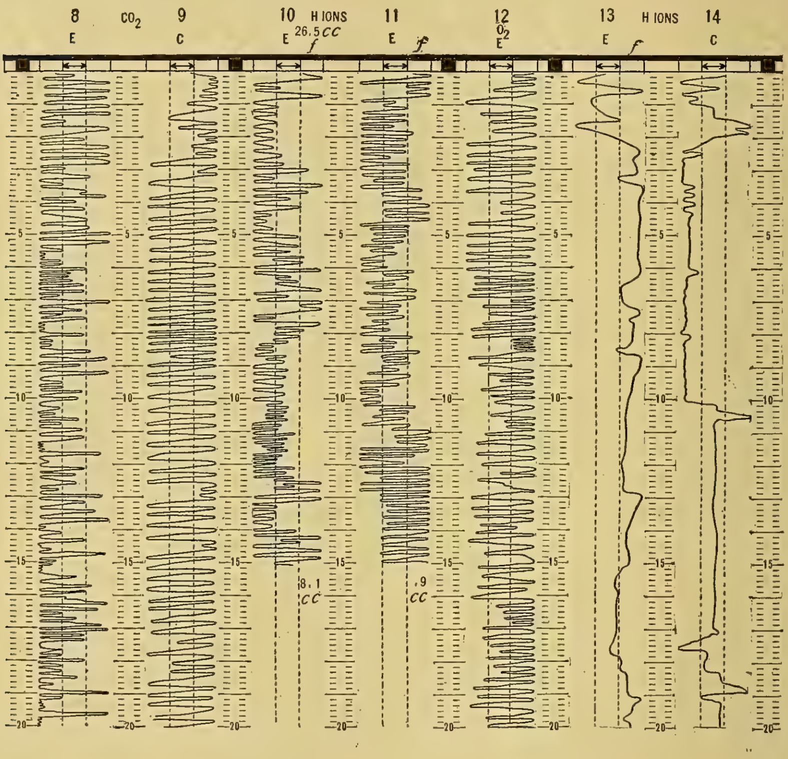

Chart II.

Graph 8 shows the avoidance of carbon dioxide in sea water introduced at the right. Graph 9 is the control of the same.

Graphs 10 and 11 show the reaction to fresh water rendered acid by the addition of 26.5 c.c. per liter of carbon dioxide and the reversal of the reaction when the carbon dioxide fell to 8.1 c.c. and finally the gradual reversal to a preference for the fresh water when it became less acid than the salt.

Graph 12 shows the preference for sea water with oxygen added (right end.)

Graph 13 shows the selection of essential neutrality by a small salmon. 14 is the control of the same.

per l. and the reaction was very sharply negative to fresh water. The concentration of the carbon dioxide in the fresh water was gradually lowered and the avoidance fell off as is shown in graph 12 which was really only a continuation of 11 interrupted to take a sample which showed the carbon dioxide content to be 8.1 c.c. per l. During the period represented by 11 the negative reaction decreased gradually until a point was reached when the tank was probably about equally acid throughout, after which the fish became negative to the sea water at the end of 13 minutes when on the basis of a uniform decrease the sea water which usually contained a little less than 2 c.c. per l., became more acid than the fresh. Thus it appears that these fish are as sensitive to acidity as litmus paper.

The relation of the two species of fishes to salinity is interesting in this connection. The salmon goes into fresh water to breed and some may reach maturity there or they may return to salt at varying ages. In connection with the entrance of salmon into fresh water, the orientation of these specimens with head in the fresh water is of interest but it is evident that the orientation is with reference to acidity and alkalinity rather than salinity. Sea water is less acid than fresh and the reactions of the salmon accord with their recent entrance into salt water. In the case of the herring, they are known to enter fresh water and some remain there permanently. Lydekker[1] states that some of them will live in brackish water and become dwarfed.

When carbon dioxide was used in sea water the avoidance of the higher concentration was very striking, in all concentrations tried, up to 70 c.c. per l. The avoidance was usually proportional to the concentration with staggering in the very high ones just as is the case with the fresh water fishes.

6. *Oxygen.*

The oxygen in the sea water in use at the station never reached saturation. One experiment was tried with water drawn directly from the tap, against water aërated by running over a board. The fishes selected the aërated water. When oxygen was added to the water used in opposition to that drawn directly from the tank the preference for the higher oxygen content was decided (graph 12).

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[1] *New N. H.*, Vol. V., p. 489.

V. Summary and Discussion of Conclusions.

In these brief experiments we have only outlined the possibilities of much more extensive work along similar lines. Such experimental study alone can of course not solve the problems of migration but the extreme sensitiveness of the fishes studied, as shown by their detection of slight deviations from neutrality, temperature differences as small as 0.2 of a centigrade degree, of small fractions of a cubic centimeter per liter of hydrogen sulfide, etc., makes it very clear that there is no difficulty in fishes determining the direction to large rivers from hundreds of miles out at sea or of finding their way into any bay or harbor or river or other arm of the sea which their particular physiological condition at a given time demands. It is not necessary to appeal to "instinct" to explain the return of certain salmon to certain rivers, or the running of herring in certain localities. The mere fact of their origin in the region, the probable limited tendency to leave it (Johnstone, '08), coupled with their ability to detect and follow slight differences in water is a sufficient explanation of all their peculiar migrations. The close way in which animals stay about certain localities from generation to generation is hardly appreciated. Thus as Johnstone points out, the herring of the east coast of Britain are largely local, having formerly been assumed to belong to shoals that came from distant points.

The experimental method cannot of course determine the cause for the absence of fishes from any given point but must be accompanied by hydrographic studies. Such combined efforts must give very trustworthy results; hydrographic studies alone may lead to entirely erroneous assumptions because of the lack of knowledge of the sensibilities of the fishes concerned and the selection of some insignificant factor correlated with their absence or presence, as an explanation. Such correlates, offered as explanations, become the basis of erroneous remedial measures.

Noting the remarkable discriminations of fishes for differences in alkalinity, acidity and neutrality, a note of warning may be sounded in regard to the relation of pollution to the run of herring, and the presence in valuable numbers of many other fishes. Their tendency to avoid acid waters, hydrogen sulfide, etc., which result from decomposition and are increased by the presence of refuse of fish canneries, sewage, etc., makes diversion of such refuse from the sea an important consideration. The Baltic towns of the Hanseatic League were dependent in part upon the herring industry and after a century of great growth and prosperity fell into decline at the middle of the fourteenth century. Their prosperity was the accompaniment of the presence of great shoals of herring off the Island of Rügen in the Baltic. Their decline was caused in part by the failure of the herring industry and the supposed migration of the herring to the North Sea which has since been the center of the industry. Schouwen (on the Netherland coast of the North sea) appears in the fourteenth century to have been frequented by the herring shoals in preference to Rügen (Yeats, '86). The rapid growth of the Netherland cities, their supremacy and final separation from the Hanseatic league followed. A little later the herring again changed their haunts choosing the coast of Norway where both Norsemen and Netherlanders caught them. The Beukelszoon method of curing herring having come into use nearness to home was no longer a necessity. The Norse fisheries flourished until 1587 when an "apparation of a gigantic herring frightened the shoals away." Thus it appears that the development of the herring industry in each locality led to the apparent desertion of the locality by the fish, though the migrations assumed by historians may be doubted (Yeats) (Putzger '01, p. 17a). Was this due to the contamination of the sea by the cities, or merely to over catch? Whichever may have been the case it is certain that contamination will not invite runs of the herring. The common assumption that the sea is so large that pollution cannot have a significant rôle is rendered entirely untenable by the greatly increased sensitiveness of the marine fishes as compared with fresh water ones.

VI. Acknowledgments and Bibliography.

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'13 Tide Tables for the Pacific Coast of the U. S. together with a number of Foreign Ports in the Pacific Ocean. Reprinted from Tide Tables for 1914. Dept. of Commerce Coast and Geodetic Survey, Washington, D. C.

Weigelt, C.

'03 L'assainessement et le repeuplement des rivieres. Memoires courounes. (Translated from German) Brussels.

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'13 The Resistance of Fishes to Different Concentrations and Combinations of Oxygen and Carbon dioxide. Biol. Bull., Vol. 25, No. 6, pp. 323–347.

'14 Resistance and Reactions of Fishes to Temperature. Transactions of the Illinois. Acad. Science, Vol. VII., 1914.

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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

Smithsonian Institution
JUN 30 1915
National Museum

VOL. XXVIII JUNE, 1915 No. 6

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Editorial Staff

E. G. Conklin—*Princeton University.*
Jacques Loeb—*The Rockefeller Institute for Medical Research*
George T. Moore—*The Missouri Botanic Garden.*
T. H. Morgan—*Columbia University.*
W. M. Wheeler—*Harvard University.*
E. B. Wilson—*Columbia University.*

Managing Editor

Frank R. Lillie—*The University of Chicago.*

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Vol. XXVIII. *June, 1915.* *No. 6.*

BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY

SEVENTEENTH REPORT

FOR THE YEAR 1914

I. TRUSTEES

EX OFFICIO

FRANK R. LILLIE, *Director*, The University of Chicago.
GILMAN A. DREW, *Assistant Director*, Marine Biological Laboratory.
D. BLAKELY HOAR, *Treasurer*, 161 Devonshire Street, Boston, Mass.
GARY N. CALKINS, *Clerk of the Corporation*, Columbia University.

TO SERVE UNTIL 1918

CORNELIA M. CLAPP	Mount Holyoke College.
E. G. CONKLIN	Princeton University.
ROSS G. HARRISON	Yale University.
CAMILLUS G. KIDDER	27 William Street, New York City.
M. M. METCALF	Oberlin College.
WILLIAM PATTEN	Dartmouth College.
JACOB REIGHARD	University of Michigan.
W. B. SCOTT	Princeton University.

TO SERVE UNTIL 1917

S. F. CLARKE	Williams College.
CHARLES A. COOLIDGE	Ames Building, Boston, Mass.
C. R. CRANE	Woods Hole, Mass., President of the Board.
ALFRED G. MAYER	Carnegie Institution.
C. E. MCCLUNG	University of Pennsylvania.
T. H. MORGAN	Columbia University.

Erwin F. Smith	United States Department of Agriculture.
E. B. Wilson	Columbia University.

TO SERVE UNTIL 1916

H. H. Donaldson	Wistar Institute of Anatomy and Biology.
M. J. Greenman	Wistar Institute of Anatomy and Biology.
C. W. Hargitt	Syracuse University.
H. S. Jennings	Johns Hopkins University.
George Lefevre	University of Missouri, Secretary of the Board.
A. P. Mathews	University of Chicago.
G. H. Parker	Harvard University.
Henry B. Ward	University of Illinois.

TO SERVE UNTIL 1915

H. C. Bumpus	Tufts College.
R. A. Harper	Columbia University.
W. A. Locy	Northwestern University.
Jacques Loeb	The Rockefeller Institute for Medical Research.
F. P. Mall	Johns Hopkins University.
George T. Moore	Missouri Botanical Garden, St. Louis.
L. L. Nunn	Telluride, Colo.
John C. Phillips	299 Berkeley Street, Boston, Mass.

II. ACT OF INCORPORATION

No. 3170.

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our LORD ONE THOUSAND, EIGHT HUNDRED and EIGHTY-EIGHT.

HENRY B. PIERCE,
Secretary of the Commonwealth.

[SEAL.]

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The Director and Assistant Director, who shall be chosen by the Trustees, shall also be Trustees, *ex officio*.

II. Special meetings of the members may be called by the Trustees, to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President and may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The President shall annually appoint two Trustees, who shall constitute a committee on finance, to examine from time to time the books and accounts of the Treasurer, and to audit his accounts at the close of the year. No investments of the funds of the Corporation shall be made by the Treasurer except approved by the finance committee in writing.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be given to the Boston Society of Natural History, or some similar public institution, on such terms as may then be agreed upon.

IX. These By-Laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-Laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. TREASURER'S REPORT

CASH RECEIPTS AND DISBURSEMENTS FOR THE YEAR ENDING DECEMBER 31, 1914

RECEIPTS

Cash on hand January 1, 1914	$ 4,663.05	
Annual dues	1,024.00	
BIOLOGICAL BULLETIN	1,221.12	
Dexter House	4,151.18	
Charles R. Crane	34,200.00	
Donations	35.14	
Dormitory, stone building	424.60	
Dormitory, Whitman Cottage	252.75	
Fish Trap	995.24	
Instruction, botany	750.00	
Instruction, embryology	1,000.00	
Instruction, physiology	500.00	
Instruction, zoology	2,100.00	
Mess	12,749.22	
Miscellaneous	620.15	
Research	2,950.00	
Supply department	14,003.35	$81,639.80

PAYMENTS

Administration	$ 7,202.56
BIOLOGICAL BULLETIN	1,771.64
Boats	5,604.42
Carpenter shop	288.27
Chemical department	2,640.20
Dexter House	4,104.87
Dormitory, stone building	204.06
Dormitory, Whitman House	148.37
Dredge	1,751.00
Fish Trap	1,206.41
Instruction, botany	950.00
Instruction, embryology	475.00
Instruction, physiology	650.00

Instruction, zoölogy	1,139.00	
Lectures	18.85	
Library	3,000.88	
Maintenance of buildings and grounds	4,573.61	
Mess	17,665.68	
Miscellaneous	1,569.59	
New laboratory (furniture and equipment)	10,263.10	
Philosophical lectures	100.00	
Pumping station	3,892.38	
Supply department	10,295.75	
Supply department improvements	437.48	
Cash on hand January 1, 1915	1,686.68	$81,639.80

Cash Receipts and Disbursements on Account of Funds, August 1, 1914 to January 1, 1915

Reserve Fund

Cash on hand August 1, 1914	$107.77	
Div. 14 shs. United Shoe Mach. Corp. Pfd.	5.25	
Div. 6 shs. Am. Smelting & Refining Co. Pfd.	21.00	
Div. 8 shs. Gen. Elec. Co.	16.00	
Interest on deposit	.28	$150.30

Lucretia Crocker Fund

Cash on hand August 1, 1914	$136.29	
Div. 1 sh. West End St. Ry. Co.	1.75	
Div. 18 shs. Vermont & Mass. Ry. Co.	54.00	
Div. 2½ shs. Gen. Elec. Co.	5.00	
Div. 1 sh. Am. Tel. & Tel. Co.	2.00	
	$199.04	
1914 scholarship	100.00	99.04

Library Fund

Cash on hand August 1, 1914	$157.79	
Div. 5 shs. United Shoe Mach. Corp. Pfd.	1.87	
Div. 1 sh. Am. Smelting & Refining Co. Pfd.	3.50	
Div. 3 shs. Am. Tel. & Tel. Co.	6.00	
Div. 2½ shs. Gen. Elec. Co.	5.00	174.16
Cash on hand January 1, 1915		$423.50

HARVEY S. CHASE AND COMPANY
Certified Public Accountants
84 State Street
Boston

December 18, 1914.

D. BLAKELY HOAR, ESQ.,
Treasurer, Marine Biological
Laboratory at Woods Hole, Mass.,
161 Devonshire Street, Boston.

Dear Sir: We have audited the accounts of the Marine Biological Laboratory as kept at Woods Hole, and of the Trust Funds and accounts as kept at your office, 161 Devonshire Street, for the year ended December 31, 1913.

We have checked the report of the Treasurer, submitted to us, and find it correct and in accordance with the books and accounts of the Laboratory. The extent of the audit is set forth in a detailed report under date of November 25, 1914.

Very respectfully,
HARVEY S. CHASE & CO.

COST OF THE NEW LABORATORY, BY CONTRACT, PAID DIRECTLY FROM MR. CRANE'S OFFICE

L. D. Willcut Sons Co., Builders	$77,810.32
C. E. Cronin, Plumbing	9,482.52
Edwin Lewis, Electrician	3,479.00
Cleghorn Heating Co., Heating	3,132.55
Otis Elevator Co., Elevator	1,485.00
Simpson Bros., Sinks	360.00
Monroe Refrigerator Co.	195.00
Architect's Commission	4,842.61
	$100,787.00

V. LIBRARIAN'S REPORT

AUGUST, 1914

All who have worked in Woods Hole for any length of time must appreciate how grateful we are to have the library so well cared for, now, in the new fireproof building. It is certainly a great relief to fear no longer its sudden destruction. In addition to this, the librarian may here announce the appointment of an assistant librarian to be in charge at the Laboratory throughout the year. This has been a pressing need for several years. It assures continuity of the work and has made possible many important improvements. We were fortunate to secure for this position Miss May E. Scott, who started work in April, after some weeks devoted to special training. In the meantime Dr. Drew supervised the moving and installation of the books in the new library.

The improvements noted below have been accomplished through Miss Scott's skillful management and very great industry. Unfortunately she has been ill since the middle of June, but her work was already so well in hand that it has been possible to continue most of it during this season. We must thank Miss Elizabeth Dunn for her valuable services in this emergency during the summer. Without her generous efforts in behalf of Miss Scott and the library we should have been seriously embarrassed. In addition to this we have been much helped by having extra assistance for two hours a day for the heavy summer routine.

The entire library has been re-accessioned, and new catalogs made. There are over 3,300 volumes exclusive of the reprints, which number over 1,500. A modern system has been introduced throughout, which will make the library much more accessible next year. Many missing parts were secured; and more than 500 volumes were bound, necessitating an extra expenditure of $500. Several notable additions have been made to the library:

The American Museum of Natural History has loaned us indefinitely a number of their duplicate sets and books which will be of great assistance. These works are not yet catalogued, but there will be over 2,500 volumes, among which is a number of useful sets of journals, and of memoirs, transactions, and proceedings, of academies, etc., hitherto not on our shelves.

The Journal of Biological Chemistry was added, the back volumes being a gift from the editors.

Dr. Beyer presented several boxes of books containing among other things a duplicate set of the *American Journal of Physiology*, and a set of the *Journal of Medical Research;* also *Zeitschrift für Hygiene*, and the *Archiv für Schiffs- und Tropenhygiene.*

Dr. Kellicott gave, in addition to his own text-books, about 100 volumes.

Dr. Otto Glaser sent a set of the *Proceedings of the Society of Experimental Biology and Medicine*, with other volumes.

Dr. Duggar has obtained a lot of much needed pamphlets missing from sets.

Mrs. Edward Gardiner added a number of volumes to her former gift.

Drs. Loeb, Minot, Hegner, McFarland, and Mrs. Agassiz and Mr. Crane presented books recently published.

The book-publishing companies have continued to present new books, Blakiston forwarding this week 14 volumes on chemistry, etc.

Stechert has inaugurated a new plan of sending books recently issued for examination. This was done at Dr. Oliver Strong's request, and has proved to be a great convenience.

Dr. H. F. Osborn gave a volume, and has turned over to us the premium on sales of one of his books. This will be continued.

Dr. E. E. Just has offered $5.00 for five years to assist us in securing more journals. Dr. Rice has promised $10 per year for the same purpose; Dr. E. B. Meigs gave $10.00 also, and the amount already on hand, announced last year for this fund, now totals $50.00, which will be expended for certain missing volumes of our files. We have acknowledged a number of reprints and books, but feel that here is a place where biologists may help us still more. A word to others and to publishers may greatly aid us.

The library has now reached a stage of stability. It is well housed, well cared for, of great use, and rapidly growing. We should be spending at least twice the present amount (that is about $1,000 a year more for books); especially for a number of journals, now much in demand. There should be a few more works of general character. Members of the corporation might greatly extend the library with a little effort. The exchange list might be further extended as soon as conditions become quiet abroad.

There is little to be added to the material equipment; beyond a truck with shelves for shifting volumes, and some suitable arrangement, possibly glass gallery platforms, to make the upper shelves readily accessible.

H. McE. Knower,
Librarian.

Aug. 11, 1914.

VI. THE DIRECTOR'S REPORT

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I have the honor to transmit herewith a report of the twenty-seventh session of the Marine Biological Laboratory, for the year 1914. The number of investigators in attendance was 128, as compared with 122 in 1913, 93 in 1912, and 82 in 1911. The number of students in the courses was 89 as compared with 69 in 1913, 67 in 1912, and 65 in 1911. The total attendance was 217 as compared with 191 in 1913, 160 in 1912, and 147 in 1911. The number of subscribing institutions was 40 in 1914, as compared with 30 in 1913, 29 in 1912, 25 in 1911, 24 in 1910, 20 in 1909, 18 in 1908, and 16 in 1907. The list is given on p. 356. Amherst College, Beloit College, Johns Hopkins University, Rutgers College, and the University of Wisconsin, are among the new subscribing institutions. The total membership of the Corporation is now 327 as against 303 in 1913. The receipts from subscribing institutions and students' fees were $7,300.00 as compared with $6,160.00 in 1913, $5,175.00 in 1912, $4,574.99 in 1911, $4,150.00 in 1910, and $3,700.35 in 1909. The receipts from the supply department were $14,003.35[1] in 1914, as compared with $14,554.90 in 1913, $13,966.35 in 1912, $10,303.61 in 1911, $9,300.58 in 1910 and $8,549.55 in 1909.

The main event of the year was the occupation and dedication of the new permanent laboratory building, the gift of the president of the board of trustees, Mr. Crane. It has satisfied the pressing need of space for investigation which has been felt so keenly in the past two or three years; it has also provided improved facilities so greatly needed for certain types of research; and the new library room contained in it has enabled us to undertake a definite policy of library expansion. The building proved to be perfectly adapted for the purposes for which it was erected.

---

[1] The business transacted in 1914 was actually $1,100 more than in 1913, but collections were slow.

The exercises in dedication of the new building held on July 10 were attended by representatives of universities, members of the staff, the investigators and students working at the Laboratory, and by many friends. The buildings were open to inspection in the morning, and demonstrations of sea-animals and of research in progress were made, and the laboratory steamer made a collecting trip. Lunch was served to the invited guests. The formal exercises were held at 2 P.M., in a tent erected for the occasion, with an attendance of about 800. The addresses were interspersed with music by the Russian Balalaika Orchestra.

Mr. C. R. Crane, president of the Board of Trustees, and donor of the building presided. In opening the exercises, he said:

"I think we have come here particularly to celebrate the wonderful spirit that is back of the Woods Hole Biological Laboratory. It is very difficult to define that spirit, but I think we all know something of it and something is also known all through the scientific world. Without that spirit no amount of bricks and mortar and organization would be of any great service, but with that spirit the laboratory has been able to accomplish a very great deal with very simple means.

"For some time back it has seemed to be worth while to give this spirit a more substantial body. This spirit, as I see it, is very much like the spirit that President Wilson speaks so much of, the spirit of freedom and of coöperation, the fundamental spirit of democracy. In giving this spirit a more substantial body, we have been very fortunate in having with us Dr. Drew. I think we are all very happy at the wonderful result of his year's work. There is a rumor in circulation around here that Dr Drew is a zoölogist. I believe that rumor has spread into the outside world, but I am very certain that we must all feel, after looking over the new laboratory, that Dr. Drew would have made his reputation as an engineer if he had a chance."

Short speeches were made by the Director, by Professor Conklin, and by the head of the U. S. Bureau of Fisheries, the Hon. Hugh M. Smith. Dr. R. M. Woodward, the Director of the Carnegie Institution of Washington, then delivered the main address on the Needs of Research. These addresses were published in full in *Science*.[1]

Extra provision was made this summer for the expected large attendance, by the lease of the Dexter House, which was run by the laboratory, and was made to pay expenses, including rent. With this increase the mess was able to accommodate the students and investigators comfortably. The Dexter House is in bad repair, and no lasting arrangement with the owner seems possible. We have therefore been casting about for other arrangements. The "Homestead" used for the help of the mess has long been overcrowded, and for some time we have regarded it as unsafe. Mr. Crane therefore offered to build on the homestead site a new dwelling house with accommodations for about forty people, which will be 10 to 12 in excess of the number employed in the Mess, and will therefore furnish some available space for women of the laboratory. This work is already nearly finished. Mr. Crane has also presented this autumn (1914) funds for improvement of the Cayadetta and for other purposes including the completion of the stone wall on the Yacht Club frontage and filling in behind it. This work is far advanced; when it is finished the building will be moved from in front of the new laboratory to the east end of the lot.

We shall thus begin the new year with most of our material needs satisfied to an extent that will probably be adequate for several years. The estimate of running expenses for 1915 shows a deficit of $20,000 above receipts, as in 1914. Mr. Crane has again most generously promised his support to this extent. This brings up again the need of an endowment, which I think we should keep constantly before us until attained. The flourishing condition of the Laboratory constitutes a strong argument for its endowment; the Laboratory represents no new experiment, but a demonstrated success, and the fulfilment of one of the greatest needs of American biology. The coöperation of forty American universities and the attendance of representatives of thirty-seven more proves that we are supplying a want that is felt by all the institutions of higher learning. The attendance of the largest body of scientific investigators ever gathered for work at one place and time also proves the magnitude of the want that we supply. Not only so but it demonstrates the great influence over the progress of research which the Marine Biological Laboratory exerts. We can feel justified in using all the means in our power to secure the funds that will place this great organization beyond the stress of ordinary vicissitudes.

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[1] Vol. XL., No. 1024, pp. 217–232.

Provision has been made for continuation of the students' courses as in 1914, with one exception. Dr. Drew has felt for some years that the burden of executive work during the summer is so great that it is undesirable for him longer to retain charge of the course in embryology. The directors have therefore requested Professor Wm. E. Kellicott, who has been associated with Dr. Drew in this course for several years, to assume charge of it. He has consented, and we may feel confident that he will maintain its best traditions. We must all feel nevertheless a sense of loss in the relinquishment by Dr. Drew of this important course. For many years in charge of the course in invertebrate zoölogy, and then of the course in embryology, Dr. Drew has impressed the lessons of our science on students to an extent which few teachers can equal. I am sure that all will join in congratulating Dr. Drew on his great success as a teacher, and in the hope that the future will yield him more leisure again to resume this cherished part of a scientist's work.

There are submitted as parts of this report lists of the staff of 1914, investigators and students in attendance, subscribing institutions, evening lectures and of members of the corporation.

I. THE STAFF

1914

FRANK R. LILLIE, DIRECTOR,

Professor of Embryology, and Chairman of the Department of Zoölogy, The University of Chicago.

GILMAN A. DREW, ASSISTANT DIRECTOR,

Marine Biological Laboratory.

ZOÖLOGY

I. INVESTIGATION

Gary N. Calkins	Professor of Protozoölogy, Columbia University.
E. G. Conklin	Professor of Zoölogy, Princeton University.
Gilman A. Drew	Assistant Director, Marine Biological Laboratory.
George Lefevre	Professor of Zoölogy, The University of Missouri.
Frank R. Lillie	Professor of Embryology, The University of Chicago.
C. E. McClung	Professor of Zoölogy, University of Pennsylvania.
T. H. Morgan	Professor of Experimental Zoölogy, Columbia University.
E. B. Wilson	Professor of Zoölogy, Columbia University.

II. INSTRUCTION

Caswell Grave	Associate Professor of Zoölogy, Johns Hopkins University.
W. C. Allee	Instructor in Biology, Williams College.
George A. Baitsell	Fellow in Zoölogy, Yale University.
Raymond Binford	Professor of Biology, Earlham College.
E. J. Lund	Bruce Fellow in Zoölogy, Johns Hopkins University.
T. S. Painter	Graduate Student, University of Würzburg.

EMBRYOLOGY

I. INVESTIGATION (See Zoölogy)

II. INSTRUCTION

Gilman A. Drew	Assistant Director, Marine Biological Laboratory.
Lorande L. Woodruff	Assistant Professor of Biology, Yale University.
William E. Kellicott	Professor of Biology, Goucher College.
Robert A. Budington	Professor of Zoölogy, Oberlin College.

PHYSIOLOGY

I. INVESTIGATION

Albert P. Mathews	Professor of Physiological Chemistry, The University of Chicago.
R. S. Lillie	Professor of Biology, Clark University.
Harold C. Bradley	Assistant Professor of Physiological Chemistry, University of Wisconsin.

II. INSTRUCTION

Ralph S. Lillie	Professor of Biology, Clark University.
Walter E. Garrey	Associate Professor of Physiology, Washington University Medical School.
Frank P. Knowlton	Professor of Physiology, Syracuse University.
Edward B. Meigs	Associate in Physiology, Wistar Institute of Anatomy and Biology.

PHILOSOPHICAL ASPECTS OF BIOLOGY AND ALLIED SCIENCES

LECTURES

Edward G. Spaulding	Assistant Professor of Philosophy, Princeton University.

BOTANY

George T. Moore	Director, Missouri Botanical Garden and Professor of Botany, Washington University.
George R. Lyman	Assistant Professor of Botany, Dartmouth College.
B. M. Duggar	Physiologist, Missouri Botanical Garden and Professor of Plant Physiology, Washington University.
Ivey F. Lewis	Assistant Professor of Botany, University of Wisconsin.
W. J. Robbins	Instructor in Plant Physiology, Cornell University.
A. R. Davis	Lackland Research Fellow, Shaw School of Botany.

LIBRARY

H. McE. Knower	Professor of Anatomy, University of Cincinnati, Librarian.
Mary E. Scott	Assistant Librarian.

CHEMICAL SUPPLIES

Oliver S. Strong	Instructor in Anatomy, College of Physicians and Surgeons, New York City, Chemist.

SUPPLY DEPARTMENT

G. M. Gray	Curator.
John J. Veeder	Captain.
E. M. Lewis	Engineer.
O. F. Curtis	Collector and Assistant Curator of Botanical Supplies.
A. W. Leathers	Collector.
A. M. Hilton	Collector.
Edna E. Wells	Clerk

F. M. MacNaught	Business Assistant.

2. INVESTIGATORS AND STUDENTS

INVESTIGATORS

1914

ZOOLOGY

Independent Investigators

Allee, W. C., Assistant Professor in Zoölogy, University of Oklahoma.
Baitsell, George A., Instructor in Biology, Yale University.
Begg, A. S., Instructor in Comparative Anatomy, Harvard Medical School.
Binford, Raymond, Professor of Zoölogy, Earlham College, Richmond, Ind.
Browne, Ethel N., 510 Park Ave., Baltimore, Md.
Budington, Robert A., Professor of Zoölogy, Oberlin College.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Chidester, Floyd E., Assistant Professor of Zoölogy, Rutgers College.
Child, C. M., Associate Professor of Zoölogy, University of Chicago.
Clapp, Cornelia M., Professor of Zoölogy, Mount Holyoke College.
Clark, Eliot R., Professor of Anatomy, University of Missouri.
Clark, Eleanor L., Johns Hopkins Medical School.
Coe, Wesley R., Professor of Biology, Yale University.
Conklin, E. G., Professor of Biology, Princeton University.
Cowdry, Edmund V., Associate in Anatomy, Johns Hopkins Medical School.
Cowdry, N. H., Johns Hopkins Medical School.
Crampton, H. E., Professor of Zoölogy, Barnard College, Columbia Univ.

Danchakoff, Wera, Director of laboratory for medical research, Moscow, Russia.
Dolley, David H., Professor of Pathology, University of Missouri.
Donaldson, H. H., Wistar Institute of Anatomy and Biology.
Drew, Gilman A., Assistant Director, Marine Biological Laboratory.
Dunn, Elizabeth H., Woods Hole, Mass.
Erdmann, Rhoda, Theresa Leesell Research Fellow, Yale University.
Goldfarb, A. J., Professor of Biology, College of the City of New York.
Grave, Caswell, Associate Professor of Zoölogy, Johns Hopkins University.
Hegner, Robert W., Assistant Professor of Zoölogy, University of Michigan.
Hogue, Mary J., Instructor in Zoölogy, Wellesley College.
Jackson, Frederic S., Lecturer in Histology and Embryology, McGill Univ.
Jordan, Harvey E., Professor of Histology and Embryology, University of Virginia.
Kellicott, W. E., Professor of Biology, Goucher College.
Kingsbury, Francis B., Instructor, University of Minnesota.
Knower, Henry McE., Professor of Anatomy, University of Cincinnati.
Kunkel, Beverly W., Professor of Zoölogy, Beloit College.
Lefevre, George, Professor of Zoölogy, University of Missouri.
Lewis, Margaret R., Johns Hopkins Medical School.
Lewis, Warren H., Professor of Physiological Anatomy, Johns Hopkins Medical School.
Lillie, Frank R., Professor of Embryology, University of Chicago.
Loomis, Frederic B., Professor of Comparative Anatomy, Amherst College.
Lund, E. J., Instructor in Protozoölogy, University of Pennsylvania.
MacDowell, E. C., Instructor, Yale University.
Malone, E. F., Assistant Professor of Anatomy, University of Cincinnati.
McClung, C. E., Director of the Zoölogical Laboratory, University of Pennsylvania.
Morgan, T. H., Professor of Experimental Zoölogy, Columbia University.
Newman, H. H., Associate Professor of Zoölogy, University of Chicago.
Nowlin, Nadine, Assistant Professor of Zoölogy, University of Kansas.
Painter, T. S., Instructor, Yale University.
Patterson, J. T., Professor of Zoölogy, University of Texas.
Pearl, Raymond, Biologist of Maine Agricultural Experiment Station.
Peebles, Florence, Lecturer, Bryn Mawr College.
Pinney, Mary E., Demonstrator in Biology, Bryn Mawr College.
Richards, A., Instructor in Zoölogy, University of Texas.
Shorey, Marian L., Professor of Biology, Milwaukee-Downer College.
Spaulding, E. G., Professor of Philosophy, Princeton University.
Stockard, Charles R., Professor of Anatomy, Cornell Medical College.
Strong, O. S., Instructor in Anatomy, Columbia University.
Strong, R. M., Instructor in Zoölogy, University of Chicago.
Tennent, David H., Professor of Biology, Bryn Mawr College.
Wieman, H. L., Assistant Professor of Zoölogy, University of Cincinnati.
Wildman, E. E., University of Pennsylvania.
Wilson, E. B., Professor of Zoölogy, Columbia University.
Woodruff, L. L. Assistant Professor of Biology, Yale University.
Zeleny, Charles, Associate Professor of Zoölogy, University of Illinois.

Beginning Investigators

Adkins, W. S., Graduate Student, Columbia University.

Allen, Ezra, Professor of Biology at Philadelphia School of Pedagogy, University of Pennsylvania.

Bridges, Calvin B., Fellow in Zoölogy, Columbia University.

Bullock, Frederick D., Instructor in Cancer Research, Columbia University.

Carothers, E. Eleanor, University of Pennsylvania.

Cobb, Margaret V., University of Illinois.

Dexter, John S., Graduate Student, Columbia University.

Field, Hazel E., Graduate Student, University of Chicago.

Goodrich, H. B., Fellow in Zoölogy, Princeton University.

Gould, Harley N., Fellow in Biology, Princeton University.

Gunther, Maude C., Instructor in Biology, Eastern High School, Washington, D. C.

Heilbrunn, L. V., Assistant in Zoölogy, University of Chicago.

Hoge, Mildred A., Graduate Student, Columbia University.

Hoy, William E., Jr., Fellow in Biology, Princeton University.

Key, J. A., Student, Johns Hopkins Medical School.

Larrabee, Austin P., Professor of Biology, Fairmount College.

Leathers, A. L., Instructor in Zoölogy, Northwestern University.

Medes, Grace, Graduate Student, Bryn Mawr College.

Metz, Chas. W., Carnegie Institution Staff, Cold Spring Harbor, L. I.

Moore, Carl R., Fellow in Zoölogy, University of Chicago.

Morris, Margaret, Osborne Zoölogical Laboratory, Yale University.

Okkelberg, Peter, Instructor in Zoölogy, University of Michigan.

Packard, Charles, Instructor in Zoölogy, Columbia University.

Shumway, Waldo, Assistant in Zoölogy, Columbia University.

Stark, Mary B., Graduate Student, Columbia University.

Stocking, Ruth J., Student, Johns Hopkins University.

Sturtevant, A. H., Graduate Student, Columbia University.

Weinstein, Alexander, University Scholar in Zoölogy, Columbia University.

Woodward, Alvalyn E., Fellow in Zoölogy, University of Michigan.

Yocum, H. B., Instructor in Zoölogy, Kansas State Agricultural College.

Young, Donnell B., Laboratory Assistant, Columbia University.

PHYSIOLOGY

Independent Investigators

Bradley, H. C., Associate Professor of Physiological Chemistry, University of Wisconsin.

Chambers, Robert, Jr., Assistant Professor of Histology and Comparative Anatomy, University of Cincinnati.

Garrey, W. E., Associate Professor of Physiology, Washington University Medical School.

Harvey, E. N., Instructor in Physiology, Princeton University.

Hyde, Ida H., Professor of Physiology, University of Kansas.

Just, E. E., Professor of Physiology, Howard University.

Kanda, Sakyo, Research Assistant, University of Minnesota.

Kite, George D., Assistant in Physiology, Henry Phipps Institute.

Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.

Lillie, R. S., Professor of Biology, Clark University.
Loeb, Jacques, Head of Department of Experimental Biology, Rockefeller Institute for Medical Research.
Mathews, A. P., Professor of Physiological Chemistry, University of Chicago.
Mattill, Henry A., Professor of Physiological Chemistry, University of Utah.
Meigs, Edward B., Fellow in Physiology, Wistar Institute of Anatomy and Biology.
Moore, A. R., Associate Professor of Physiology, Bryn Mawr College.
Oliver, Wade W., Student, Ohio-Miami Medical College.
Tashiro, Shiro, Instructor in Physiological Chemistry, University of Chicago.
Uhlenhuth, Eduard, Rockefeller Institute for Medical Research.
Warren, Howard C., Stuart Professor of Psychology, Princeton University.
Wasteneys, Hardolph, Associate in Experimental Biology, Rockefeller Institute for Medical Research.
Werber, Ernest I., Instructor in Anatomy, Northwestern University.
Wherry, W. B., Associate Professor of Bacteriology, University of Cincinnati.

Beginning Investigators

Hyman, Libbie H., Laboratory Assistant in Zoölogy, University of Chicago.

BOTANY

Independent Investigators

Blakeslee, A. F., Professor of Botany and Genetics, Connecticut Agricultural College.
Colley, R. H., Instructor in Botany, Dartmouth College.
Davis, A. R., Lackland Research Fellow, Shaw School of Botany, Washington University.
Duggar, B. M., Physiologist in charge of Graduate Laboratory and Professor of Plant Physiology, Missouri Botanical Garden.
Gates, R. R., University of London, England.
Lewis, I. F., Professor of Botany, University of Missouri.
Lyman, George R., Assistant Professor of Botany, Dartmouth College.
Moore, Geo. T., Director, Missouri Botanical Garden.
Rumbold, Caroline, 3824 Locust St., West Philadelphia, Pa.
Stokey, Alma G., Associate Professor of Botany, Mount Holyoke College.

Beginning Investigators

Stewart, Mary W., Assistant in Botany, Barnard College, Columbia Univesity.
Sweatman, Elizabeth A., 3032 Parkwood Ave., Toledo, Ohio.
Wuist, Elizabeth D., Instructor in Botany, Milwaukee State Normal School.

STUDENTS

1914

ZOOLOGY

Allen, William Ray, Assistant in Kansas State Agricultural College, Manhattan. Kans.
Applegate, Anne G., Student, Western College, Oxford, Ohio.

Applegate, Eleanor, Western College, Oxford, Ohio.
Ashman, Richard, Student, Rutgers College, New Brunswick, N. J.
Ballou, Marion M., Mount Holyoke College.
Batchelder, Charles H., Assistant, New Hampshire College, Durham, N. H.
Behre, Charles H., Jr., Student, New Orleans High School.
Carroll, Mitchell, 617 South 16th St., Philadelphia.
Childs, Henry E., Student, University of Rochester.
Clark, Katharine E., Assistant in Zoölogy, Mt. Holyoke College.
Closson, J. Harwood, 53 W. Chelten Ave., Germantown, Pa.
Cohn, Edwin T., University of Chicago.
Connet, Helene, Student, Goucher College, Baltimore.
Dickinson, Claire, Teacher in elementary schools, New York University.
Elliott, Marguerite, Vassar College.
Greene, Phillips F., Biology Laboratory Assistant, Amherst College.
Greene, Walter F., Amherst College.
Halsted, Margie H., Instructor in Biology, West High School, Rochester, N. Y;
Hamilton, Florence N., Student, Vassar College.
Jackson, Frances E., Student, Mt. Holyoke College.
Janney, Marion, Goucher College.
Kellogg, Emilie, Student, Mt. Holyoke College.
Ladoff, Sonia, Assistant in Biology, State Normal School, La Crosse, Wis.
Lancaster, DeWitt B., College of Charleston, S. C.
Loeb, Robert F., Student, University of Chicago.
Lovett, J. Elizabeth, Student, Goucher College.
Mannhardt, L. Alfred, Student, Yale University.
McWilliams, Minnie R., Student, University of Chicago.
Montgomery, Priscilla B., 105 S. 41st St., Philadelphia.
Moses, Bessie L., Student, Goucher College.
Mullikin, Jeannette, Vassar College.
Pappenheimer, Beatrice L., 309 W. 99th St., New York City.
Place, Jesse A., Instructor in Biology, Ohio University, Athens, Ohio.
Redfield, Elizabeth S., Student, Radcliffe College.
Rones, Marguerite T., Teacher in elementary schools, Boston.
Rush, J. E., Assistant Professor, Wisconsin.
Schmoll, Hazel M., Assistant in Biology, Vassar College.
Stocking, Besse E., Student, Goucher College.
Strongman, Bessie T., Student, University of Colorado.
Thomas, Anna M., Student, Carnegie Institute of Technology.
Tiesing, Paul E., Student, Yale University.
Warren, Catherine C., Princeton, N. J.
Wayman, Marguerite, Student, Hunter College, New York City.

EMBRYOLOGY

Allen, Charles E., Assistant Instructor, Wabash College.
Baldwin, Francis M., Instructor in Biology, Western Maryland College.
Binkley, Lelia T., University of Texas.
Chamberlain, Mary M., Student, Bryn Mawr College.
Davis, Carl L., Professor of Anatomy, George Washington University, Washington, D. C.
Diehl, Jane K., Student, Wellesley College.

GOVER, MARY, Goucher College, Baltimore, Md.
HESS, WALTER N., Instructor, Pennsylvania State College, State College.
HUNTER, OSCAR B., Professor of Histology and Embryology, Associate in Anatomy, George Washington University, Washington, D. C.
MANN, MARY LEE, Barnard College.
MCFARLAND, HELEN J., Student, Bryn Mawr College.
MILLS, FRANCES A., Barnard College.
NELSON, THURLOW C., Assistant in Zoölogy, University of Wisconsin.
OGDEN, WARNER, Student, Carleton College, Northfield, Minn.
PLOUGH, HAROLD H., Assistant in Zoölogy, Amherst College.
POTTER, BESS, Student, Doane College, Crete, Neb.
PREBLE, JESSIE L., Student, Bryn Mawr College.
TAFT, ANNIE E., Curator Dept. of Neuropathology, Harvard Medical School.
WALTON, ARTHUR C., Student Assistant, Northwestern University.
WARE, CLARA C., Graduate Student, Columbia University.
YOUNG, THOMAS O., Student, Carleton College, Northfield, Minn.

PHYSIOLOGY

ATWOOD, WARREN G., Student, Dartmouth College.
BENJAMIN, BLANCHE M., Student, Radcliffe College.
FREDERICK, NORA, Teacher in Biology, Lewis Institute, Chicago.
HYMAN, LIBBIE H., Laboratory Assistant in Zoölogy, University of Chicago.
LINTON, EDWIN S., Graduate Student, Washington and Jefferson College.
LYNCH, VERNON, Graduate Student, Johns Hopkins University.
OLIVER, SYMMES F., University of Michigan, Ann Arbor.
REDFIELD, ALFRED C., Assistant in Zoölogy, Harvard University.
VANNEMAN, AIMÉE S., Vassar College.
WALLING, LALIA V., Instructor in Physiology, University of Kansas.

BOTANY

ALLARD, ANNE D., Teacher in Boston Normal School, Boston.
BURKS, GEORGE PAUL, Student, Wabash College.
CARROLL, FRANKLIN B., 617 South 16th St., Philadelphia.
COBB, RUTH, Smith College.
FINE, SOLOMON, Student, Rhode Island State College, Kingston, R. I.
FORBES, WILLIAM T. M., 23 Trowbridge Road, Worcester, Mass.
FRITZ, CLARA W., McGill University.
GREEN, NEWTON B., Student, Oberlin College.
HERRICK, JOSEPH C., Professor of Biology, St. Joseph's Seminary, Yonkers, N. Y.
MALLARD, AGNES K., Teacher, Boston Normal School, Boston.
MCLAUGHLIN, FREDERICK A., Instructor in Botany, Mass. Agricultural College.
SEVERY, J. WARREN, Student, Oberlin College.
STEARNS, FRANCES L., Teacher, Central High School, Grand Rapids, Mich.
WANN, F. B., Missouri Botanical Garden, St. Louis, Mo.
WILLEY, ALBERT G., Student, Dartmouth College.

3. TABULAR VIEW OF ATTENDANCE

	1911	1912	1913	1914
INVESTIGATORS—Total	82	93	122	128
Independent:				
Zoölogy	42	44	58	62
Physiology	18	14	17	22
Botany	8	10	11	10
Under Instruction:				
Zoölogy	12	21	21	31
Physiology	2	2	7	1
Botany		2	7	3
STUDENTS—Total	65	67	69	89
Zoölogy	26	24	33	43
Embryology	20	15	22	21
Physiology	6	11	8	10
Botany	13	17	7	15
TOTAL ATTENDANCE	147	160	191	217
INSTITUTIONS REPRESENTED—Total		57	80	77
By investigators	37	43	50	51
By students	31	36	41	47
SCHOOLS AND ACADEMIES REPRESENTED				
By investigators	3	2	3	1
By students	9	1	6	5

4. SUBSCRIBING INSTITUTIONS

AMHERST COLLEGE.
BARNARD COLLEGE.
BELOIT COLLEGE.
BRYN MAWR COLLEGE.
CARLTON COLLEGE.
COLUMBIA UNIVERSITY.
CARNEGIE INSTITUTE OF TECHNOLOGY.
DARTMOUTH COLLEGE.
DOANE COLLEGE.
GOUCHER COLLEGE.
HARVARD UNIVERSITY.
HARVARD MEDICAL SCHOOL.
HUNTER COLLEGE, N. Y. C.

KANSAS STATE AGRICULTURAL COLLEGE.
JOHNS HOPKINS UNIVERSITY.
LUCRETIA CROCKER SCHOLARSHIPS.
MCGILL UNIVERSITY.
MOUNT HOLYOKE COLLEGE.
NORTHWESTERN UNIVERSITY.
OBERLIN COLLEGE.
PRINCETON UNIVERSITY.
RADCLIFFE COLLEGE.
RHODE ISLAND STATE COLLEGE.
ROCHESTER UNIVERSITY.
ROCKEFELLER INST. FOR MED. RESEARCH.
SMITH COLLEGE.
RUTGERS COLLEGE.
UNIVERSITY OF CHICAGO.
UNIVERSITY OF CINCINNATI.
UNIVERSITY OF ILLINOIS.
UNIVERSITY OF KANSAS.
UNIVERSITY OF MICHIGAN.
UNIVERSITY OF PENNSYLVANIA.
UNIVERSITY OF WISCONSIN.
VASSAR COLLEGE.
WELLESLEY COLLEGE.
WESTERN COLLEGE.
WISTAR INSTITUTE.
WABASH COLLEGE.
YALE UNIVERSITY.

5. EVENING LECTURES, 1914

Friday, July 3,
PROF. T. H. MORGAN.........“Chromosomes and Mendelian Heredity.”

Tuesday, July 7,
DR. ERWIN F. SMITH.........“Crown Gall in Plants, with Reference to the Nature and Origin of Cancer.”

Tuesday, July 14,
DR. G. L. KITE..............“The Colloidal Structure of Living Matter as Determined by Microdissection.”

Friday, July 17,
Dr. Lawrence J. Henderson . . "The Functions of the Environment."

Tuesday, July 21,
Prof. D. H. Tennent . . . . "Hybridization in Sea-urchins."

Friday, July 24,
Prof. H. C. Warren . . . . "Freedom of Teaching in American Colleges."

Tuesday, July 28,
Dr. Frank M. Chapman . . . . . . "An Ornithological Expedition to Colombia."

Friday, July 31,
Dr. Raymond Pearl . . . . "The Physiology of Reproduction in the Domestic Fowl."

Tuesday, Aug. 4,
Dr. Alfred G. Mayer . . . . "The Coral Reefs of Torres Straits."

Friday, Aug. 7,
Dr. Edwin Linton . . . . "Reminiscences of the Woods Hole Laboratory of the U. S. Fish Commission, 1882–1889."

Friday, Aug. 14,
Dr. R. R. Gates . . . . "Recent Aspects of Mutation."

6. MEMBERS OF THE CORPORATION.

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Md.
Billings, Mr. R. C., 66 Franklin St., Boston, Mass.
Carey, Mr. Arthur Astor, Fayerweather St., Boston, Mass.
Clarke, Prof. S. F., Williams College, Williamstown, Mass.
Conklin, Prof. Edwin G., Princeton University, Princeton, N. J.
Crane, Mr. C. R., Woods Hole, Mass.
Davis, Major Henry M., Syracuse, N. Y.
Evans, Mrs. Glendower, 12 Otis Place, Boston, Mass.
Farlow, Prof. W. G., Harvard University, Cambridge, Mass.
Fay, Miss S. B., 88 Mt. Vernon St., Boston, Mass.
Folsom, Miss Amy, 88 Marlboro St., Boston, Mass.

Foot, Miss Katherine, 80 Madison Ave., New York City, N. Y.
Gardiner, Mrs. E. G., Woods Hole, Mass.
Gardiner, Miss Eugenia, 15 W. Cedar St., Boston, Mass.
Hannaman, Mr. Charles E., 103 1st St., Troy, N. Y.
Harrison, Ex-Provost C. C., University of Pennsylvania, Philadelphia, Pa.
Jackson, Miss M. C., 88 Marlboro St., Boston, Mass.
Jackson, Mr. Chas. C., 24 Congress St., Boston, Mass.
Kennedy, Mr. Geo. G., 284 Warren St., Roxbury, Mass.
Kidder, Mr. C. G., 27 William St., New York City, N. Y.
Kidder, Mr. Nathaniel T., Milton, Mass.
King, Mr. Chas. A.,
Lee, Mrs. Frederic S., 279 Madison Ave., New York City, N. Y.
Lowell, Mr. A. Lawrence, 171 Marlboro St., Boston, Mass.
Marrs, Mrs. Laura Norcross, 9 Commonwealth Ave., Boston, Mass.
Mason, Mr. E. F., 1 Walnut St., Boston, Mass.
Mason, Miss Ida M., 1 Walnut St., Boston, Mass.
Means, Mr. James Howard, 196 Beacon St., Boston, Mass.
Merriman, Mrs. Daniel, Worcester, Mass.
Minns, Miss Susan, 14 Louisburg Square, Boston, Mass.
Minns, Mr. Thomas, 14 Louisburg Square, Boston, Mass.
Mixter, Miss M. C., 241 Marlboro St., Boston, Mass.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Sts., New York City, N. Y.
Morgan, Prof. T. H., Columbia University, New York City, N. Y.
Morgan, Mrs. T. H., New York City, N. Y.
Noyes, Miss Eva J., 28 South Willow St., Montclair, N. J.
Nunn, Mr. Lucian L., Telluride, Colo.
Osborn, Prof. Henry F., American Museum of Natural History, New York.
Phillips, Dr. John C., Windy Knob, Newham, Mass.
Phillips, Mrs. John C., Windy Knob, Newham, Mass.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pa.
Pulsifer, Mr. W. H., Newton Center, Mass.
Rogers, Miss A. P., 5 Joy St., Boston, Mass.
Sears, Dr. Henry F., 420 Beacon St., Boston, Mass.

Shedd, Mr. E. A.,
Smith, Mrs. C. C., 286 Marlboro St., Boston, Mass.
Strobell, Miss E. C., 80 Madison Ave., New York City, N. Y.
Thorndike, Dr. Edward L., Teachers College, Columbia University, New York City, N. Y.
Trelease, Prof. William, University of Illinois, Champaign, Ill.
Ware, Miss Mary L., 41 Brimmer St., Boston, Mass.
Warren, Mrs. S. D., 67 Mt. Vernon St., Boston, Mass.
Whitney, Mr. Henry M., Brookline, Mass.
Willcox, Miss Mary A., Wellesley College, Wellesley, Mass.
Wilmarth, Mrs. H. D., Elliott St., Jamaica Plain, Mass.
Williams, Mrs. Anna P., 505 Beacon St., Boston, Mass.
Wilson, Dr. E. B., Columbia University, New York City, N. Y.
Wilson, Prof. W. P., Philadelphia Museum, Philadelphia, Pa.

2. Members, January, 1915

Abbott, Prof. J. F., Washington University, St. Louis, Mo.
Abbott, Miss Margaret B., The Bennett School, Milbrook, N. Y.
Addison, Dr. W. H. F., University of Pennsylvania, Medical School, Philadelphia, Pa.
Adkins, Mr. W. S., Texas Christian University, Fort Worth Texas.
Allee, Dr. W. C., University of Oklahoma, Norman, Okla.
Allen, Prof. Ezra, 413 Lancaster St., Ardmore, Pa.
Allyn, Miss Harriet M., Hackett Medical College, Canton, China.
Alsburg, Dr. C. S., U. S. Dept. of Agriculture, Washington, D.C.
Baitsell, Dr. George A., Sheffield Scientific School, Yale University, New Haven, Conn.
Baker, Dr. E. H., 5436 University Ave., Chicago, Ill.
Bancroft, Prof. F. W., Aloha Farm, Concord, California.
Bardeen, Prof. C. R., University of Wisconsin, Madison, Wis.
Beckwith, Miss Cora J., Vassar College, Poughkeepsie, N. Y.
Behre, Miss Elinor H., Sophie Newcomb College, Tulane University, New Orleans, La.
Beyer, Dr. H. G., Stoneleigh Court, Washington, D. C.
Bigelow, Prof. M. A., Teachers College, Columbia University, New York City, N. Y.

BIGELOW, PROF. R. P., Mass. Institute of Technology, Boston, Mass.

BINFORD, DR. RAYMOND, Earlham College, Richmond, Ind.

BINKLEY, MISS LELIA T., University of Texas, Austin, Texas.

BLAKESLEE, PROF. A. F., Connecticut Agricultural College, Storrs, Conn.

BOX, MISS CORA MAY, University of Cincinnati, Cincinnati, Ohio.

BRADLEY, DR. HAROLD C., University of Wisconsin, Madison, Wis.

BROWNE, MISS ETHEL N., East Hall, Univ. of California, Berkeley, Cal.

BUDINGTON, PROF. R. A., Oberlin College, Oberlin, Ohio.

BUMPUS, DR. H. C., Tufts College, Mass.

BYRNES, DR. ESTHER F., 193 Jefferson Ave., Brooklyn, N. Y.

BUCKINGHAM, MISS EDITH N., 342 Marlboro St., Boston, Mass.

CALKINS, PROF. GARY N., Columbia University, New York City, N. Y.

CALVERT, PROF. PHILIP P., Univ. of Pennsylvania, Philadelphia, Pa.

CARLSON, PROF. A. J., University of Chicago, Chicago, Ill.

CARVER, MR. GAIL L., 307 Adams St., Macon, Georgia.

CARY, DR. L. R., Princeton University, Princeton, N. J.

CATTELL, PROF. J. MCKEEN, Garrison-on-Hudson, N. Y.

CHAMBERS, DR. ROBERT, JR., University of Cincinnati, Cincinnati, Ohio.

CHESTER, PROF. WEBSTER, Colby College, Waterville, Me.

CHIDESTER, DR. F. E., Rutgers College, New Brunswick, N. J.

CHILD, PROF. C. M., University of Chicago, Chicago, Ill.

CLAPP, PROF. CORNELIA M., Mount Holyoke College, South Hadley, Mass.

CLARK, DR. E. R., University of Missouri, Columbia, Mo.

COE, PROF. W. R., Yale University, New Haven, Conn.

COLLEY, DR. R. H., Dartmouth College, Hanover, N. H.

COLTON, PROF. H. S., Ardmore, Pa.

COOLIDGE, MR. C. A., Ames Bldg., Boston, Mass.

COPELAND, DR. MANTON, Bowdoin College, Brunswick, Maine.

COWDRY, DR. E. V., Johns Hopkins Medical School, Baltimore, Md.

Crampton, Prof. H. E., Barnard College, Columbia University, New York City, N. Y.

Crane, Mrs. C. R., Woods Hole, Mass.

Curtis, Prof. W. C., University of Missouri, Columbia, Mo.

Derick, Prof. Carrie M., McGill University, Montreal, Canada.

Dexter, Mr. J. S., Oliver College, Olivet, Mich.

Dodds, Prof. G. S., University of Missouri, Columbia, Mo.

Donaldson, Prof. H. H., Wistar Institute of Anat. and Biol., Philadelphia, Pa.

Dorrance, Miss Ann, Dorranceton, Pa.

Dorrance, Miss Frances, Dorranceton, Pa.

Drew, Prof. Gilman A., Marine Biological Laboratory, Woods Hole, Mass.

Duggar, Prof. B. M., Missouri Botanical Garden, St. Louis, Mo.

Dungay, Dr. Neil S., Carleton College, Northfield, Minn.

Eaton, Prof. E. H., Hobart College, Geneva, N. Y.

Edwards, Dr. D. J., College of the City of New York, New York City, N. Y.

Eigenmann, Prof. C. H., University of Indiana, Bloomington, Ind.

Ewald, Dr. W. F., Kaiserin Augustastr. 78, Berlin, W 10, Germany.

Farnam, Miss Louise W., 43 Hillhouse Ave., New Haven, Conn.

Ferguson, Prof. J. S., Cornell Univ. Medical School, New York City, N. Y.

Field, Miss Hazel E., University of Chicago, Chicago, Ill.

Field, Prof. Irving, Auburn, Mass.

Fish, Mr. J. Burton, 883 Freeman St., New York City, N. Y.

Flanigen, Miss Ruth, Woodbury, N. J.

Gage, Prof. S. H., Cornell University, Ithaca, N. Y.

Garrey, Prof. W. E., Washington University Medical School, St. Louis, Mo.

Gies, Prof. W. J., Columbia Univ., Dept. Physiological Chemistry, New York City, N. Y.

Glaser, Prof. O. C., University of Michigan, Ann Arbor, Mich.

Glaser, Dr. R. W., Bussey Institution, Forest Hills, Mass.

Goldfarb, Prof. A. J., College of the City of New York, New York City, N. Y.

GOODRICH, MR. H. B., Princeton University, Princeton, N. J.

GRAVE, DR. CASWELL, Johns Hopkins University, Baltimore, Md.

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HAHN, DR. C. W., High School of Commerce, New York City, N. Y.

HALL, PROF. ROBERT W., Lehigh University, South Bethlehem, Pa.

HANCE, MR. ROBERT T., Univ. of Cincinnati, Cincinnati, Ohio.

HARGITT, DR. C. W., Syracuse University, Syracuse, N. Y.

HARMAN, DR. MARY T., Kans. State Agricultural College, Manhattan, Kans.

HARPER, PROF. R. A., Columbia University, New York City, N. Y.

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HARVEY, PROF. B. C. H., University of Chicago, Chicago, Ill.

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HAUGHWONT, MR. F. G., Columbia University, New York City, N. Y.

HAYDEN, MISS MARGARET A., Carnegie Inst. of Technology, Pittsburgh, Pa.

HAYES, PROF. S. P., Mount Holyoke College, South Hadley, Mass.

HEATH, PROF. HAROLD, Stanford University, San Francisco, Cal.

HEGNER, PROF. R. W., University of Michigan, Ann Arbor, Mich.

HEILBRUNN, MR. L. V., University of Chicago, Chicago, Ill.

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REGENERATIVE POTENCIES OF DISSOCIATED CELLS OF HYDROMEDUSÆ.

CHAS. W. HARGITT.

INTRODUCTORY.

At various times during my earlier work on the development and regeneration of hydromedusæ, especially that dealing with the developmental capacity of egg fragments, there had grown the conviction of the remarkable potencies of the various tissue elements of these organisms. So strong had this impression become that the desirability of repeating Trembly's picturesque experiment of turning *Hydras* inside out and testing again the possibilities that ectoderm and entoderm might really exchange functions under the new conditions involved was entertained. While admitting the rather convincing results of the experiments of Ischikawa ('90), touching this feature it still seemed that there might be some warrant that under certain conditions Trembly's conclusions might find confirmation. However, the writer never found the convenient season for trying out the experiment, though other correlated features were observed at various times in connection with the work above cited, especially in the summer of 1908 while working on the development of *Clava* and *Hydractinia*. This was further incited by the work of H. V. Wilson, "On Some Phenomena of Regeneration in Sponges," '07. Therefore with the opportunity for investigation at the Naples Laboratory I set about a series of experiments with a view to settle some of the problems concerned. My work at Naples began in December, 1910, and continued till mid-April following, and during this period systematic experiments were made upon the regenerative potencies of somatic cells of about a dozen different species, among which the following may be named: two species of *Eudendrium*, two species of *Tubularia*, one each of *Hydractinia*, *Podocoryne*, *Campanularia*, *Obelia*, *Halecium*, *Sertularia*, and a medusa, *Liriope exigua*.

In *Science* of March 10, 1911, appeared a preliminary report of similar experiments by Dr. Wilson, which reached Naples about April 1, just when my own experiments were being concluded, results of which had already been written and some of which will appear in the following sections just as originally prepared. I immediately wrote Professor Wilson, giving a brief account of my work and stating that my results would be held pending the appearance of his completed report. This appeared in due course ('11). While dealing in the main with different material yet he had employed essentially the same methods which I had followed and they seemed so conclusive that I had laid my own paper aside, deeming it unnecessary. However, the report in the *Journal* of the Marine Biological Association of similar experiments by DeMorgan and Drew (Oct., '14) which seemed to express some doubt as to the conclusiveness of Wilson's results, prompts me to submit even at this late date my own results, though in somewhat abbreviated form.

Material and Methods.

Concerning material employed in the experiments mention has been made in the previous section as to the several species used, though two species of medusa instead of one were experimented upon. For the most part particular care was taken to have perfectly fresh and vigorous specimens, but it was later found that this precaution was not absolutely essential in all cases, some of my best hydranths having been reared from material which had been several days in the laboratory before being used. Later mention will also be made of a probable reason why this may happen. One point however calls for special emphasis, namely, that of the freshness and purity of the water used in the experiments. In my work several expedients were employed to guard against the presence of parasitic organisms, especially predatory protozoa. Of most efficiency was that of having water fresh from the open sea. Another expedient was that of sterilizing water of the aquaria, and still a third was that of using synthetic, that is, artificial sea-water. But of all these the first was found to be most satisfactory.

Concerning methods much might be said, though only the briefest reference will be made of those employed by me. Among the several modes of isolating the tissue cells the following were employed: With hydroids the cœnosarc may be forced out of the perisarc by clipping off the hydranths and then stripping the stems through clean fingers, or the ends of smooth forceps, or similar device. Again, one may finely clip up the stems with scissors and then still further reduce the cells by continuing the operation in deep watch glasses or small beakers. A still further mode was used, that of clipping up the stems with scissors and later grinding the tissues under a smooth glass rod whose end had been rounded in the flame, employing it as a pestle and a watch glass as a mortar. One objection to this was the crushing of the individual cells in many instances, and otherwise injuring them. On the other hand there may be reason to believe that such treatment is not really so serious an injury as might at first sight appear, for as will be seen in some later discussion, the shock may actually serve as a stimulus to hasten cellular despecialization and hence initiate regenerative processes.

Cell dissociation having been effected the next method is to arrange them in dishes where aggregation may take place. First in this operation is the straining or filtering out of debris and such fragments as are undesirable. This was done chiefly by pressing the cells through bolting cloth. In my experiments it was found that a better medium than silk bolting cloth was a fine meshed cheese cloth, or a coarse meshed linen or cotton fabric, which was softer and apparently more efficient. In a few cases in the earlier experiments I merely placed the entire mass in watch glasses and with a pipette carefully drew off most of the coarser stuff and left the cells as free as such a process might leave them. On the whole, the pressing process worked better and was more expeditious. Following this operation the cells were left for a time to settle and then the milky sediment was carefully drawn off, when fresh water was added and the dishes set aside in bowls surrounded by running water to ensure as constant a temperature as could be had.

The Experiments.

Podocoryne carnea.—This was the first species which came to hand and proved one of the most responsive and convincing of the entire series. Several colonies were brought in, all occupying shells inhabited by hermit crabs, the usual habitat of this species. In one respect the species is rather difficult to operate on owing to the spinous condition of the basal cœnosarc, which made it hard to obtain enough of the polyps to make the desired culture. By allowing the colony to expand fully in shallow dishes it was possible by a dextrous sweep of the scissors to cut off quite a bunch at a single time, and by allowing others to expand in the same way and repeat the operation it was found possible to secure sufficient material for the culture. The specimens were finely cut or ground into a pulpy mass, filtered through the sterilized cloth, and thus fitted to undergo later changes. It may as well be stated here that among hundreds of preparations relatively few gave completely successful results in the regeneration of new polyps. My first surprise was not that many of the preparations "went bad," but that any survived the operation and went forward in regeneration. Here as in most experiments on regeneration a large mortality occurs in the preparations.

Character of the Dissociated Cells.—If examined soon after their dissociation one may easily distinguish the several sorts of cells even under a magnification of three hundred diameters, that is, ectoderm, entoderm, nematocyst, interstitial, etc. The very minute ectoderm cells are in striking contrast with the large flagellated cells of the entoderm. In the course of an hour, sometimes less, these differences become less marked, and ultimately almost disappear. They have become despecialized into potentially embryonic cells, and probably from this change have acquired their regenerative capacities. A careful study of such dissociated cells from various species has strongly suggested the probability that some such cytomorphic process is involved in most regenerative phenomena, and leaves little doubt that the features under consideration here are positively brought about through such a process.

Cell Aggregation.—Examination of a culture within a few hours, three to five, will show that a remarkable change has taken place among the cells in their relations to each other. They will be found to have formed numerous small nodular groups having the appearance in many cases of embryonic morulæ, or blastulæ. It was this phenomenon among others already mentioned which first raised the question in my mind as to their regenerative possibilities many years ago. Concerning the mode by which this process of aggregation is brought about there is some doubt. The attempt was made to actually observe it by carefully keeping a fresh culture under direct observation with the microscope. It was thought that the action of the flagellated cells of the entoderm might act as a means by causing vortices in the water, but careful study failed to show that this was a factor of any direct value. Such action of these cells may be easily seen but its effects are as often repellent as attractive. The fact that certain of the cells show amœba-like aspects suggested a possible amœboid action in the process. But here again no evidence whatever was found to prove the suggestion. One might imagine some chemotropic influence, but no evidence was found that such was the case. I am inclined to the view that chance contact is perhaps the chief factor in the process. This is made probable by the fact that such aggregation may be greatly facilitated by mechanical agitation of the cells, and by a gentle rotary motion of the dishes. In the earlier experiments considerable care was taken to handle the dishes as little as possible during the early stages of an experiment, thinking such might be undesirable, but later the opposite view was taken, and the dishes often rotated to hasten the process. It must be admitted, however, that there seemed to be other factors involved, for even when a considerable mass of cells had been brought together by this means there was later found to have been a sort of segregative process at work, for the mass had been more or less broken up into sections or lobes which later behaved as entirely independent bodies.

The cell aggregates, while rather predominantly sub-spherical in shape, showed considerable variation. Some were flattish, or disk-like, and some were somewhat lobulated and irregular in shape. But throughout a series of such aggregates one of the most conspicuous features was that already referred to above, namely, the resemblance to an embryonic blastula or morula, especially a hydroid morula; and if one were to take account of such morulæ as those of *Pennaria* or *Turritopsis* or *Hydractinia* it would include practically the entire range of shape exhibited by these regeneration aggregates, and one might designate them as regeneration morulæ, for such they really seem to be.

Encystment.—Following the process of aggregation there occurred in those vitally active a process of encystment, that is, the secretion of a definite perisarc about the entire mass, and its adhesion to the bottom of the glasses. Lest this feature be regarded as peculiar to these particular cases it should be pointed out that the phenomenon is often shown at a certain stage in the normal development of the hydromedusæ, and indeed in some scyphomedusæ as well. The writer has directed attention to this in the case of *Cyanea* ('02, '10) and it is doubtless shared by many others. Its function is doubtless protective, just as is that of the perisarc in the adult hydroid. Encystment usually occurs shortly following the completion of the phase of aggregation just described. This encysted stage may continue for an indefinite time, or it may be of short duration. The latter was more frequently the case with *Podocoryne* than with some others. In the present case the cyst was frequently ruptured for the upgrowth of the hydranth within a comparatively short time, say two days; but in many cases this stage persisted for a week or even more, and indeed in certain cases the cyst became a prison, being so dense as to become impenetrable from within as well as without. This again is comparable with what may happen in such stages in normal development (vide supra). This process of perisarc formation often takes various forms, following the phases of growth. In *Podocoryne* there was frequently the development of a reticulated hydrorhiza before the appearance of a hydranth, and later there appeared nodular enlargements of these stolon-like tubes and from these points would occur the upgrowth of a series of polyps. In one such preparation I obtained three vigorous young hydranths.

What has been stated in this connection as to *Podocoryne* is likewise true of other species experimented with. The behavior of the encysted aggregation morulæ is quite like that of the growing stolons of the hydrorhiza. Both may live for weeks under these conditions without any signs of further development.

Again, after such prolonged periods there may come about another direction of regenerative activity and a hydranth may arise. Aside from the evidence of life observed in the active circulation within the cœnosarc of stolons it becomes easy for one to recognize in the character of the cells of the various structures the evidences of life or death, and furthermore death of any portion is rapidly followed by disintegration brought about by microörganisms. It should have been stated in the earlier part of this section that the process of encystment usually begins soon after the aggregation phase is complete, which may be within twelve to twenty hours, though it may not become evident until much later, thirty to forty hours. The first evidence of its formation is the adhesion of the mass to the bottom of the glass, and somewhat later may be distinguished as a very delicate transparent film covering the entire mass. Its later extension may be easily followed as the growth of stolons takes place, which may be quite rapid in some cases, or in others very slow. Here again as was pointed out in an earlier connection, there is a marked similarity in the aspects of regenerative growth and those of embryonic development which further emphasizes the probability that they are fundamentally identical, having their initiative in potentially embryonic cells.

Polyp Formation.—In *Podocoryne* the first evidence of definitive hydranth organization was found during the second day following the experiment. This consisted in the dissolution of the cyst at its upper surface and the protrusion of a bud-like upgrowth. At first these were barely distinguishable, but during the third day they had become large enough to be seen with the unaided eye. The first fully formed hydranth appeared early on the fifth day, when a polyp having the distinctive form of hypostome and three tentacles was noted. This was followed by further growth of the young specimen in all its parts. The movement of the tentacles and their growth in length was interesting and striking, leaving not the least doubt as to the genuineness of the regenerative process. Usually the first three tentacles appeared at about the same time, but in a few cases it was noted that when first observed there were but two, though a third appeared rather soon after. The full six tentacles of the new polyp were developed within the next two or three days, and conformed exactly to the phases of the growth of an embryonic specimen. It should be emphasized in this connection that in the rate of growth in these specimens, as in the entire regenerative process, there was great individual difference. Apparently this was dependent upon the state of vitality of the underlying organization. For example, it was found that development was slower, and the resulting polyps smaller when arising from small cell aggregates, and in cases where there had been an excessive stolonization prior to polyp formation. In the one case it would seem as if the store of energy was small to begin with, and in the second that it had been depleted by excessive stolon formation.

The young polyps continued to live for several weeks, much longer than would have seemed probable when the highly artificial conditions, and the very limited food supply are taken into consideration. During the course of the experiments more than a dozen of these polyps of *Podocoryne* were reared to functional maturity and many others to such stage as to leave no trace of doubt as to the validity of the results.

Let it be remarked here that in this species all the material was in the asexual condition, that is, there were polyps only, no signs whatever of medusæ, which are the sexual stage in the life cycle of *Podocoryne*. Other experiments go to show that so far as use of material of asexual or mixed condition no difference as to regenerative potency could be distinguished. In *Eudendrium* where the medusa stage is absent, and where one finds sex cells in various stages of growth, the experiments were apparently not thereby influenced at all. Indeed, in those cases in which egg cells were present they took no part whatever in later regenerative activity, either degenerating or being absorbed as yolk material.

Eudendrium.—In experiments upon *Eudendrium* two species were used, *E. rameum*, and *E. racemosum*, both very common at Naples. Methods of treatment were the same as in the case already described. The promptness with which these hydroids had responded in the numerous previous experiments by the writer[1] and others in regeneration and regulation led me to anticipate that a similar type of reaction might be anticipated in this connection, but as will be seen this expectation was not realized fully. The early reactions in aggregation, encystment, etc., were quite as prompt and promising as in *Podocoryne*. And in these features the species showed nothing peculiar. But beyond the initial stages the results were disappointing. The mortality was much greater and the growth reactions much less energetic. Experiments were varied in every way practicable, hydranths alone being used for obtaining disorganized cells, cœnosarc alone, male colonies alone and female colonies alone. There seemed to be no very marked differences in results, though the cells obtained from crushing hydranths gave the least satisfactory results. As already stated the early stages followed quite as in *Pocodoryne*, encystment, and stolonization, but beyond these my experiments were far less satisfactory than in the former. In only a few cases was I able to obtain polyps, and these were small and very weak. A few developed tentacles, but never the usual number, nor were they more than buds on the base of the hydranth. The few polyps which developed secreted the usual perisarc, which was indistinguishable from that of an embryonic *Eudendrium*.

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[1] Biol. Bull., Vol. I., p. 35.

Tubularia.—As in the former I employed two species, *T. mesembryanthemum*, and *T. larynx*. As in the former the early reactions were prompt and quite like the others. But unlike the others my experiments never afforded a single polyp. The massing of dissociated cells was quite as prompt and the resulting morula-like embryo as promising as in either of the others. The encystment of perisarc followed in due order, and these lived for many days, but they never showed further signs of development. Perhaps no hydroid genus has had so large a place in experimental work as has *Tubularia*. If therefore my anticipations as to the behavior in cellular regeneration of *Eudendrium* were disappointing, those concerning *Tubularia* were really perplexing, at least for the time being. I think an explanation may be ventured which, though not absolutely convincing, may relieve a measure of the perplexity. It is to the effect that regenerative potency in an organism is more or less conditioned by its state of vitality, or in still more suggestive phrase, its *physiological* *state*, at the time it is subjected to the test. This has been recognized in principle in experiments on ordinary regenerative processes, in that only especially vigorous specimens are used. Further discussion of this point will be deferred to another section of the paper.

Other Species.—As indicated in the outstart, about a dozen different species were tried in the course of the investigation. Among these were several campanularian hydroids and two species of medusæ. The hydroids tested gave the same initial responses as those just described for *Tubularia*, but beyond that the results were likewise negative. In all cases the phase of cell-aggregation was essentially the same as in the former cases. The same was likewise true of the internal organization of the morula-like embryo, and in the perisarc formation, but beyond this there was no development.

Species of sertularian hydroids and also of *Halecium* were tested and gave exactly the same initial responses, including encystment of the embryonic mass which lived for a time but soon showed signs of disintegration and death. The reactions of the last species were the least satisfactory of any tested.

Medusæ.—Two species of medusæ were tried, though with hardly any hope of getting any regenerative responses. They were prepared just as had been the hydroids, strained through the bolting cloth and set aside after addition of fresh water. An examination of the dissociated cells showed about the same condition of the other preparations, and further inspection in about an hour showed a series of the most beautiful cell-aggregates found in any of the experiments. When it is recalled that Medusæ represent the most highly specialized group of Hydrozoa it will seem strange to find cells thus organized after having been dissociated in the manner indicated. In all my observations upon cœlenterate development I have seldom seen more typical blastula-like embryos than those under review. Unless one were actually aware of their source he could hardly have been convinced that they were not genuine embryos in process of development. However, so far as my experiments show the regenerative process does not go farther. Moreover, the organism thus formed is very short lived, and devoid of further significance so far as our problem is concerned.

ADDENDA AND DISCUSSION.

As mentioned in the introduction the immeditae occasion leading to the publication of this paper at this time after having been laid aside for four years was the appearance in the *Journal* of the Marine Biological Association, October, 1914, of a paper by DeMorgan and Drew, setting forth the results of similar experiments, all of which had given generally negative results. Moreover, certain of their conclusions seemed to leave a measure of doubt concerning the conclusiveness of certain of Wilson's experiments, and phases of their discussion involved assumptions which are at variance with those which my own work had rendered very convincing.

In the first place I desire to refer briefly to Wilson's methods and results with most of which my own are in accord. His experiments on *Eudendrium* seem to have been much more successful than my own, for which I am very glad, since it confirms with great certainty points which in my own experiments were incomplete, though sufficiently complete to warrant definite conclusions. In another point Wilson's work goes beyond my own, namely, in the admirable demonstration which his actual sections of various stages affords as to the precise features involved in the regenerative process at given times. Furthermore, the excellent series of drawings and photographs illustrating his results leave nothing to be desired in that respect, and I am purposely omitting any of my own, the only series of which not better covered are those relating to *Podocoryne*, and in these nothing essentially different occurs.

The work of DeMorgan and Drew covered experiments on two species of *Antennularia* and are restricted to these only. In order to consider certain of their views it may be well to first quote certain specific statements in their own words. "Our results largely bear out his (Wilson) contentions, though we were not successful in carrying the regenerative process as far as the production of new hydranths, and the histological structure of the restitution masses we obtained differed in many ways from that described in Wilson's paper. These differences are probably due to the fact that we experimented with other species of hydroids to those used by Wilson. The especial interest of our investigations lies in the rather anomalous fact that we have not been successful in obtaining regeneration of the complete organism from the dissociated cells. In our experiments the restitution masses, by some rearrangement or metaplastic process taking place among their conglomerated cells, formed tissue aggregates histologically reduplicating the structure of the parent organism, but in a *quite irregular and apparently meaningless manner*."

Two features in this quotation call for brief consideration, that included in the first sentence, and that which I have italicized in the last. It will have been noted in the accounts given in the earlier sections of this paper that I have given a number of cases comprising the exact equivalent of the failure they mention. This point will be further noted in a later paragraph. To the second feature it is only necessary to state that in normal hydroid development the *entire process is often "quite irregular and apparently meaningless*," frequently more so than they found in the cases concerned. In a final paragraph the authors say: "Our experiments have resulted in the production of masses that are *certainly abnormal and pathological*, but nevertheless we would submit that the segregation and rearrangement of the cells after isolation, and the comparatively long duration of life of the tumor-like masses to which they give rise are facts of considerable theoretical interest."

In this quotation I have italicized the points to which it seems necessary to make some reference. It may be admitted that in some sense such restitution masses are *abnormal*, in that the very process by which their dissociation was brought about was presumably abnormal. But that the resulting restitution masses, involving as they have the regenerative potencies of the component cells, is abnormal I must seriously challenge. Again, the assumption that they are *pathological* I should emphatically doubt. The writer once submitted a series of preparations of embryological material to a well-known cytologist and received the (at that time) very disconcerting comment, "your preparations appear to have been made from pathological material." Yet from that very material I had been getting living embryos by the hundred! So in the present case to designate as pathological cell aggregates which are producing right along perfectly normal and healthy polyps is to use a term whose significance implies the very opposite. It is admitted in the above citation that the tumor-like masses continued to live for a long time, as much as sixty days according to a preceding sentence, which shows a degree of vitality greater than that of colonies of the hydroid when placed in the aquarium. This fact of itself should prompt serious hesitation as to an assumption of a pathological condition.

It may throw some light upon the problem if attention is directed to conditions involved in the life history of many of these organisms. It is well known that many hydrozoa have alternating periods of activity and repose—growth, reproduction, etc., followed by corresponding periods of decline and more or less degeneration. In some these periodic alternations are correlated with seasonal changes in which temperature is an important factor. In others it is directly correlated with reproductive activities and has apparently little relation to season or temperature. What is of immediate importance in this connection is the fact of rather evident degenerative phases. For example, it is well known that in the spring, following the active reproductive period in several species of *Tubularia*, there is a marked degenerative phase, first evident in the casting off of the hydranths of almost the entire colony, then the gradual disintegration of the whole trophosome, till within a period of a few weeks it is difficult to find an entire and vigorous vegetative colony. An examination of the histological condition of the degenerative cœnosarc reveals the fact of positive decline marked by cytolytic conditions which might really be designated as pathologic for the time being. But even here a continued study would probably reveal the fact of its being associated with perfectly normal cyclic phases of life, being in fact phases of varying *physiological states* to which reference has already been made in an earlier connection. Similar facts of degeneration phases have also been described as associated with regenerative activity in hydroids. In experiments on *Tubularia* Stevens states in so many words: "The red granules seen in the circulation of regenerating pieces of *Tubularia* are derived from the disintegrating entodermal ridges, and are ejected by the young hydranth soon after it emerges from the tube. They are waste material rather than formative substance" ('01, p. 414). The writer himself has made similar observations in several cases, and has demonstrated the regressive condition in the cœnosarc of hibernating specimens.

A most interesting study of a series of changes of apparenly-similar character has been made by Schultz, "Über Hungererscheinungen bei *Hydra fusca*" ('06), discussed under the larger topic of "Reductionen," under which are considered a series of marked phenomena observed in organisms of various grades of complexity, including *Planaria*, *Lumbricus*, *Æolosoma*, etc.

Similar experiments by Greeley ('03), on "Effects of Variations of Temperature on Animal Tissues," show essentially the same phenomena. Among these experiments some made on *Hydra* are especially pertinent in this connection. To quote, "It was at once observed that whenever a *Hydra* is exposed to a temperature of 4° to 6° C. the tentacles gradually become thicker and shorter, and finally are completely absorbed into the body. As the absorption goes on, the ectoderm and entoderm cells of the tentacles lose their individuality and form an undifferentiated mass of protoplasm, which is slowly resolved into the body of the *Hydra*. The tentacleless body of the *Hydra* becomes slowly resolved into a dense spherical mass of coagulated protoplasm, in which no distinction between individual cells can be made out, and remains in this condition as long as it is kept at a low temperature, but quickly forms tentacles and a double layer of cells again when it is returned to the temperature of the room" (p. 43).

Enough has now been said to show, I think, that only in some qualified sense can one use such terms as *irregular*, *meaningless*, *abnormal*, *pathological*, etc., in describing phenomena such as those involved in the experiments and results under review.

As a final note it may be stated that in my experiments no attempt was made to detach and isolate the several cell-aggregates such as was done by Wilson. Neither did I attempt to augment the masses by artificially bringing several masses into contact as he had done. Attention has already been directed to the fact that there was some evidence that from larger masses were derived larger polyps, and that below a certain minimum size there was no evidence of growth.

It need hardly be stated that my experiments add little essentially new to those of Wilson; yet they seem to afford valuable confirmation of some importance which may add to the conclusiveness of his admirably conceived and conducted research. I desire also to express my gratification in the experiments of DeMorgan and Drew, which seem to me to have been admirably done and contribute to the value of the investigation as a whole.

Syracuse University,
Jan. 20, 1915

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THE EYES OF CAMBARUS SETOSUS AND CAMBARUS PELLUCIDUS.[1]

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(Department of Biology, Drury College.)

Cambarus setosus Faxon inhabits the caves of southwestern Missouri. It was described by Faxon in Garman's account of the "Cave Animals of Southwestern Missouri." An account of the eyes of this species was published by Parker.

In June, 1911, I secured an abundance of fresh material which made a reëxamination of the eyes desirable. I found it in the caves about Springfield, Sarcoxie and Ozark, Missouri. From Smallen's cave near Ozark I secured seventy-five specimens from 20 to 110 mm. in length. In addition to these, twenty-seven specimens of *C. pellucidus testii* Hay were taken from Mayfield's cave near Bloomington, Indiana. Also thirty-three *C. pellucidus* (Tellkampf) from Shawnee cave, Indiana University farm, near Mitchell, Indiana. The specimens from Indiana were collected during the fall and winter of 1911 and 1912. The blind crayfish from Indiana are smaller than those from Missouri. The smallest taken from Indiana caves were 9 mm. in length. Others varied from 15 to 60 mm. The largest specimen of *C. setosus* taken was 120 mm. in length.

C. setosus has been found only west of the Mississippi river, while *C. pellucidus* has been found only east of the Mississippi both north and south of the Ohio river. *C. pellucidus testii* Hay has been recorded only from Mayfield's Cave, Bloomington, Indiana. *C. pellucidus* has probably the widest distribution of any of the blind crayfish. It has been taken from Mammoth and other caves of Kentucky and from Wyandotte and other caves in Crawford county, from Clifty cave, Washington county, from Lost river, Orange county, from Shiloh, Down's, Donnehue's and Donnelson's caves in Lawrence county, Indiana. Strong's, Truitt's, Kuntz's, Marengo, Little Wyandotte or Seibert's and several smaller caves of Indiana were examined for blind crayfish but none were found.

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[1] Contribution from the Zoölogical Laboratory of Indiana University, No. 146. This work has been carried on under the direction of Professor Carl H. Eigenmann, to whom I am thankful for advice and criticism.

The caves of Indiana are in the same general geological formation as those of Missouri. For an account of the Missouri caves see Shepard, in the Missouri Geological Survey, Vol. XII., 1898. For the fauna and geology of Indiana caves see Green, Indiana Academy of Science, 1908, and Blatchley, Indiana Department of Geology and Natural Resources, Twenty-first Annual Report, 1896.

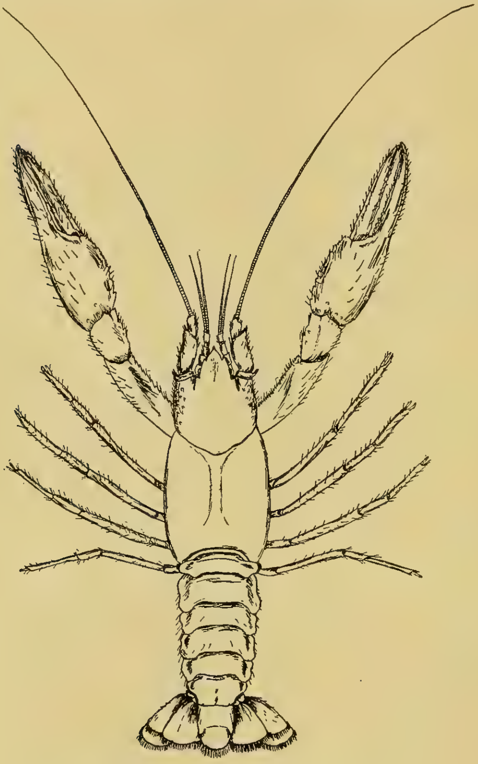
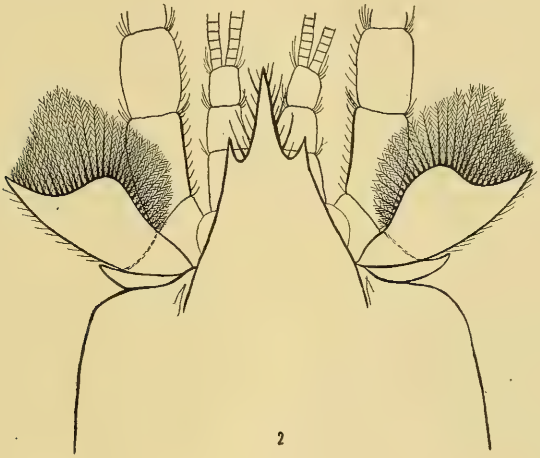


FIG. 1. Dorsal view of *C. setosus* 110 mm. long, with small, inconspicuous eyes extending slightly from under the rostrum. (Life size—reduced ½.) Drawn by Miss Dorothy L. VanDyke.

The structure of the eyes in the blind crayfish, *C. pellucidus*, was first noted by Newport (1855). Concerning his specimens which were taken from Mammoth cave, Kentucky, he states:



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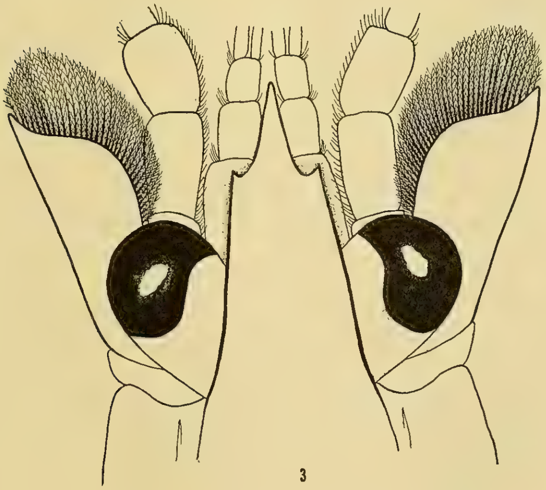

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FIGS. 2 AND 3. Dorsal views of the anterior ends of *C. setosus* and *C. propinquus*(?) 36 mm. long. They show the relative size and conspicuousness of the eyes of the two species.

"The hardened tegument which clothes the entire organ is thinnest and most transparent in that part of the eye which forms the cornea in other Crustaceans; so that the eye may be unfitted for distinguishing form, the creature may yet possess the faculty of perceiving the small amount of actinic rays of light which might penetrate into its subterranean abode . . . ; the cornea also exhibits an appearance of being divided into a few imperfect corneals (facets) at the apex of the organ, and the structures behind these into chambers, to which a small but distinct optic nerve is given." He also noted that the eyes are not pigmented.

I find that the eyes of *C. pellucidus* from Indiana caves and of *C. setosus* from Missouri caves show neither "corneals" (facets) nor "chambers, to which a small but distinct optic nerve is given."

The next writer on the eyes of blind crayfish was Leydig (1883). He stated that the cornea in *C. pellucidus* is lamellated, without pigment and without facets. His description of the internal structures of the eye is very general and indefinite.

Packard (1888) in his memoir on "The Cave Fauna of North America" describes and illustrates the form and structure of the eyes of *C. pellucidus* from Indiana and Kentucky caves and *C. hamulatus* Cope and Packard from Nickajack cave, Tennessee. He found that in both species the cornea is without facets and that the hypodermis is of the same thickness in the retinal region as in other parts of the eye; also that the optic nerve and optic ganglion are present.

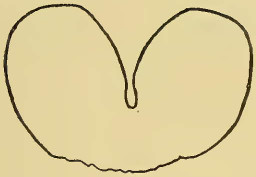
The following year (1889) Garman published Faxon's description of *C. setosus* to which reference was made in the opening paragraph.

Parker (1890) published a paper on "The Eyes in Blind Crayfishes." He had access to *C. hamulatus* and *C. setosus*. The major part of his paper deals with *C. setosus*. He emphasizes the uniform thickness of the cuticula, the nearly uniform thickness of the hypodermis and the relation of the size and conical shape of the optic stalk to the amount of degeneration, as well as the histological structure of the degenerated eye. He also called attention to the relation of the axis of the cone, which is the terminal part of the optic stalk, to the axis of the stalk itself.

The most striking characteristics of the gross anatomy of the eyes of *C. setosus* and *C. pellucidus* are smallness, lack of pigmentation and inconspicuousness. These features are shown in Figs. 1 and 2.

The eyes of all the blind crayfish examined are nearly covered by the rostrum, from a dorsal view, while the eyes of normal crayfish are only slightly concealed by the rostrum. However the eyes of the young blind crayfish are relatively larger than they are in the adult. The relative size of the eyes of *C. setosus* and *C. propinquus* (?) is shown in Figs. 2 and 3.

Size and pigmentation make the eyes of normal crayfish conspicuous. The eyes of blind crayfish are smaller and without pigment. The relative shortness of the optic stalk in the eyes of blind crayfish tends to make them inconspicuous.



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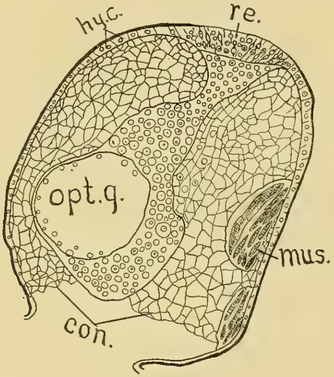

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FIG. 4. Outline of the eyes of *C. pellucidus* 10 mm. long.

FIG. 5. Horizontal (longitudinal) section of the eye of *C. setosus*, with the cuticula removed.

The distal end of the optic stalk of the eyes of the blind crayfish examined is roundish or almost hemispherical in shape. Fig. 4 which shows this was made with the aid of a camera lucida, from a fresh specimen of *C. pellucidus*, 10 mm. long. The same general form of the eye is shown in photomicrographs *A* and *B*, which were made from a horizontal section of the eyes of *C. setosus*. In no case out of fifteen series of sections of the eyes of *C. setosus* and *C. pellucidus* have I found the exaggerated conical form figured by Packard and by Parker. Crayfish killed in Perenyi's fluid and kept in 85 per cent. alcohol for a few weeks show considerable shrinkage of the connective tissue and after some months the cuticula shrinks. Dehydration and embedding also cause further shrinking. See photomicrographs *A* and *B*. The optic stalk may then approach the conical form observed by Packard and by Parker.

The cuticula of the eyes of *C. setosus* and *C. pellucidus* is usually smooth. Sometimes it is wrinkled by the fixer or preservative. These wrinkles may have led Newport to the conclusion that it is faceted. The main points of interest concerning the cuticula are; first, it is thinnest in that part of the optic stalk occupied by the cells of the vestigeal eye; second, it is laminated.

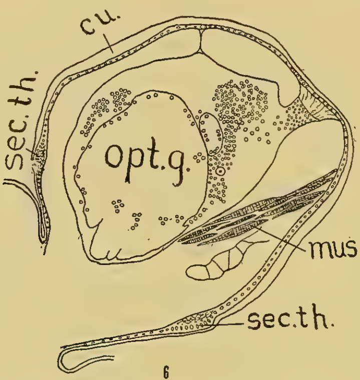

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FIG. 6. Horizontal section of the eye of *C. setosus*, with the cuticula removed Made from a section near the one shown in photomicrograph *A*.

Sometimes the cuticula in *C. setosus* and in *C. pellucidus* is from two to three times as thick on the sides as it is on the anterior end or retinal region of the optic stalk. My photomicrographs *A* and *B* and Fig. 6 show that the cuticula is thinnest in the retinal region. According to Gilbert the average thickness of the retinal cuticula is 3.41 mm., while that of the sides of the stalk is 12.41 mm. This is quite contrary to Parker's statement that "The optic stalk is covered with a cuticula which is of uniform thickness."

It will be recalled that the cuticula is secreted by the hypodermis. The hypodermis which is of ectodermal origin becomes differentiated into the visual organ in Arthropoda. This differentiation consists of the thickening and invagination of the hypodermis.

The part of the degenerated eye of the blind crayfish which is of chief interest is the retinal hypodermis. Here the largest amount of degeneration has taken place. The optic ganglion, optic tract and brain show little or no signs of degeneration. The condition of these structures is well illustrated by photomicrographs *B*, *C* and *D*.— The optic nerves (Fig. 8 and photomicrographs *C* and *E*) connecting the retinular cells with the optic ganglion are also present but they are not as well developed as the above mentioned structures.

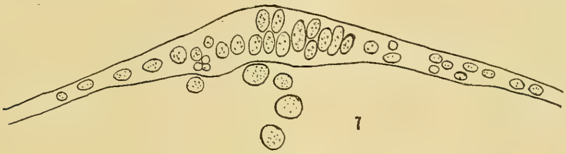

FIG. 7. A secondary thickening of the hypodermis from the antero-median part of an eye of *C. setosus*.

It is probable that when the embryology of the eye of the blind crayfish is worked out, we may be able to definitely identify the retinal cells shown in Fig. 8. The eyes of a *C. setosus* 15 mm. long show about the same amount of degeneration as the eyes of adults. The arrangement and general appearance of the retinal cells of the hypodermis, shown in Fig. 8, is comparable to an early embryonic condition found in the developing eyes of many Crustacea, the bee and other Arthropoda.

Parker states that the hypodermis in *C. setosus* is "very nearly uniform in thickness." I find that the retinal hypodermis in *C. setosus* and *C. pellucidus* is quite irregular as to thickness. Sometimes there are as many as three and four different thickened regions in a single section. These thickened places in the hypodermis are found at various places around the anterior end and the sides of the optic stalk. The principal and most common thickening is in the anterio-lateral part of the optic stalk, as is shown in Fig. 5 and photomicrographs *B*, *C* and *E*. Secondary thickenings are sometimes low on the sides of the optic stalk, as illustrated by Fig. 6 and photomicrograph *A*. Fig. 7 represents a type of the secondary thickenings. It was taken from a portion of the hypodermis along the anterio-median part of the right eye of *C. setosus*. The most highly developed or the least degenerated of these thickenings are in the anterio-lateral or retinal portion of the optic stalk. This point is well illustrated in Figs. 5 and 8 and photomicrographs *B*, *C* and *E*.

The retinal region of the hypodermis has two or three distinct layers of cells, while the rest of the hypodermis has only one layer of cells, except in secondary thickenings such as are shown in Figs. 6 and 7 and photomicrograph *A*. The cells of the retinal hypodermis in *C. setosus* and *C. pellucidus* differ in size, shape and staining properties from the other cells of the hypodermis. My drawings and photomicrographs show that the hypodermis is considerable thicker in the retinal region than elsewhere. Here again I differ from Parker who said of the hypodermis in *C. setosus:* "At least it is not thicker in the region of the retina than at many other places."

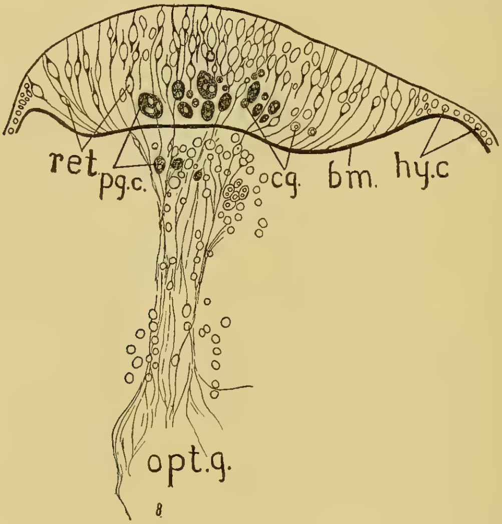

FIG. 8. Cell structure of the retinal region of the eye of *C. setosus* from a section similar to those of photomicrographs *B*, *C* and *E*.

The ommatidium or ocellus (Fig. 9) is the unit of structure of the compound eyes of Arthropoda. The functional eye of *Cambarus* has four layers of cells in the developing retinal hypodermis; the corneagen, the vitrellæ, the retinulæ and pigment cells.

The diopteric structures of the eye, such as the lens, cone and rhabdom are absent in the eyes of *C. setosus* and *C. pellucidus*. Some of the sensory cells are present even though they may be no longer functional. I believe we may feel reasonably sure of the identity of some of the cells found in these degenerated eyes.

In Fig. 8 there are at least three kinds of cells shown; (1) the large, oval, dark staining, granular cells with several nuclei, the "granular bodies," "degenerated representatives of the cones in the normal eyes" of Parker, (2) elongated cells or nuclei with fibers, and (3) small, round, granular cells with clear nuclei.

The large, oval, dark staining, granular cells with several nuclei are probably pigment cells as they are the only similar cells found on both sides of the basement membrane in the functional eyes as well as in these degenerated ones. Parker found "granular bodies" on the distal side only of the basement membrane and called them "degenerated representatives of the cones in the normal eye."

The elongated cells with fibers compare with the retinulæ of the functional eye. The retinulæ are the only cells with fibers in retina.

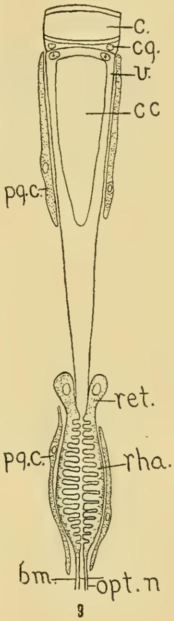

FIG. 9. The ommatidium of *Cambarus*. (After Watase.)

The small, round, granular cells with clear nuclei may be degenerated cone cells or the "undifferentiated hypodermal cells" of Parker. But most of these cells are too far removed from the cuticula to be "undifferentiated hypodermal cells" which have secreted the cuticula. The cuticula is thin in the retinal region. According to Watase, the sensory cells of the ommatidium secrete the cuticula. I am inclined to believe that the small, round, granular cells are degenerated cone cells.

Comparing the sections of the eyes of *C. setosus* and of *C. pellucidus* it is found that the eyes of *setosus* are little if any more degenerated than those of *pellucidus*. Irregularities are common in the retinal hypodermis of the eyes of these crayfish.

Whether the eyes of the blind crayfish have passed through a higher stage of development and then degenerated or whether their development has been arrested at this stage, can be determined only by a study of the developing eyes.

However I believe that the degenerated eyes of *C. setosus* and *C. pellucidus* are instances of arrested development rather than examples of degeneration. The reasons for this belief are that the eyes as found in the adult have a cell structure which appears to be comparable to the developing eyes of Crustacea. The radiate arrangement of the retinal cells of the hypodermis, is suggestive of the developing ommatidium. Also the eyes of the young blind crayfish, *C. setosus* and *C. pellucidus*, show about the same amount of degeneration as the eyes of the adult.

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EXPLANATION OF FIGURES AND ABBREVIATIONS.

The tissue was fixed with Perenyi's fluid. The sections from which the drawings and photomicrographs were made were cut eight microns in thickness and stained with Haidenhain's iron hæmatoxylin. The drawings were made with the aid of a camera lucida.

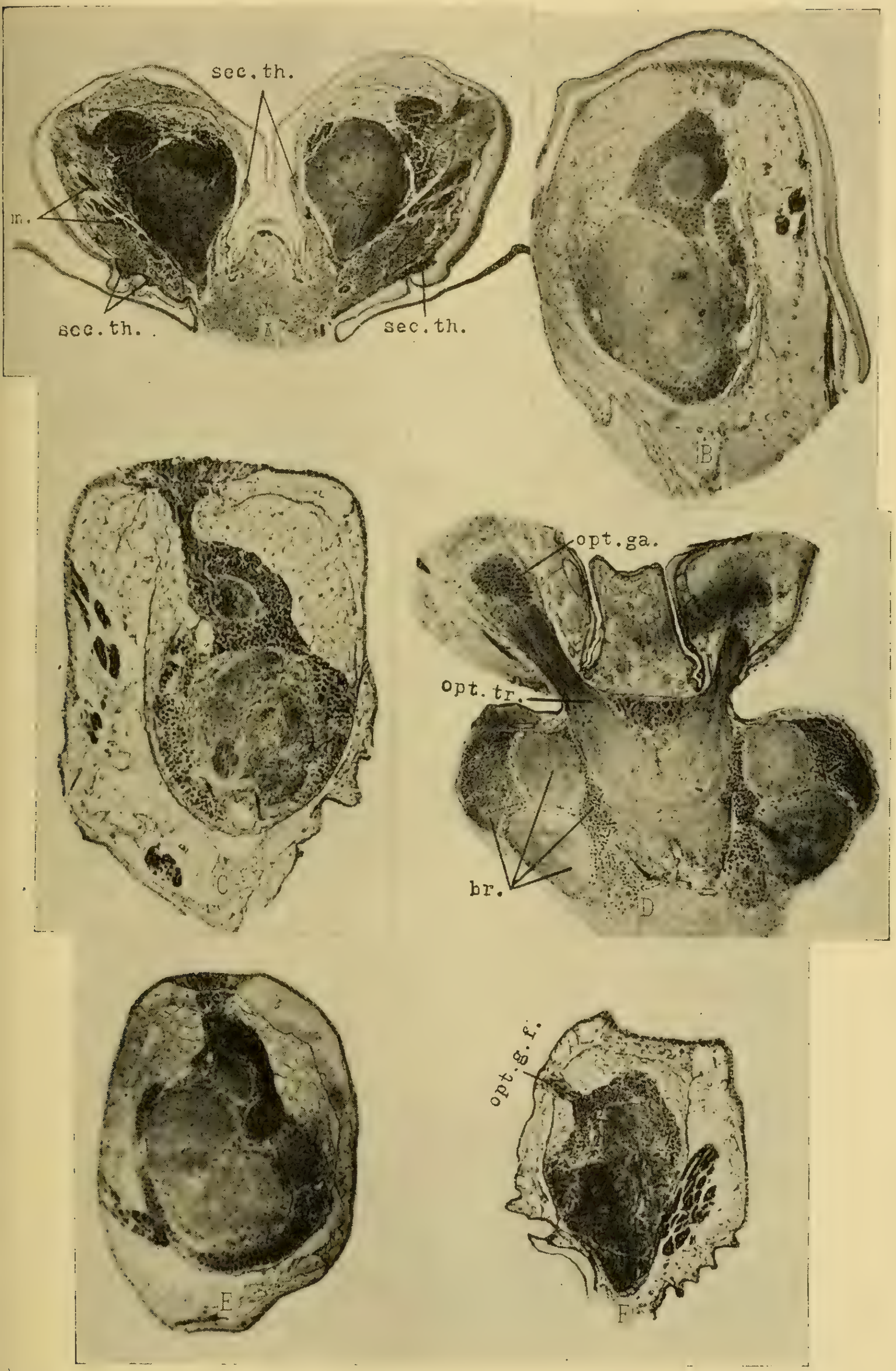
bm., basement membrane.
br., brain.
c., cornea.
cc, crystalline cone.
cg., corneagen cell.
con., connective tissue.
cu., cuticula.
hy. c., hypodermal cells.
mus., muscle.
opt. g., optic ganglion.
opt. g. f., optic ganglion fibers.
opt. n., optic nerve.
opt. tr., optic tract.
pg. c., pigment cell.
re., retina.
ret., retinulæ.
rha., rhabdom.
sec. th., secondary thickenings of the hypodermis.
v., vitrella.

PHOTOMICROGRAPH *A*. Horizontal section of the eyes of *C. setosus.*

PHOTOMICROGRAPHS *B* AND *C*. Horizontal sections of the eyes of *C. setosus. C* has the cuticula removed. The cuticula in *B* is thinner in the retinal than it is on the sides of the optic stalk. It is not as thick as it appears to be in the retinal region, due to the fact that it is wrinkled. It is also pulled loose from the underlying hypodermis, caused by the shrinking of the more delicate tissues.

PHOTOMICROGRAPH *D*. Horizontal section of the brain of *C. setosus.*

PHOTOMICROGRAPHS *E* AND *F*. Horizontal sections of the eyes of *C. pellucidus.* The cuticula has been removed. *F* has a tract of nerve fibers extending anteriomedianly towards the hypodermis, but there is no thickened portion of the hypodermis with which it is connected.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STUDIES ON TISSUES OF FASTING ANIMALS.

S. MORGULIS, PAUL E. HOWE AND P. B. HAWK.

The changes rendered in the finer structure of tissues of fasting animals have been extensively studied and the results of these investigations have an important bearing upon our understanding of the inanition phenomena in general. Apart from the interest which the subject presents from a purely histological point of view, it throws light on many obscure problems regarding the transformation of materials within the organism occasioned by the fast.

The account here presented is based upon an examination of tissues from several dogs and one fox which had died of protracted inanition, having previously suffered a very large loss in body weight. These animals had been used in a number of metabolism experiments[1] conducted some years ago in the University of Illinois.[2] The tissues were removed immediately after the animal's death and fixed in Teleschnitzky's and Zenker's fluids. The material was carried through graduated alcohols and then preserved in eighty per cent. alcohol. It was embedded in paraffin, sectioned and stained in Delafield's hæmotoxylin, with eosin as a counterstain.

A superficial examination of the sectioned material, except in a few instances, reveals nothing abnormal. But a little attentive study is sufficient to appreciate the different ways in which the effect of prolonged inanition is stamped upon the histological elements of the organism.

Looking at the smooth muscles in the intestinal tract of every one of the animals which died of fasting the cells appear turbid and without a trace of longitudinal fibrillation. The

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[1] Howe, Mattill and Hawk, *Jour. Biol. Chem.*, 10, 417, 1911 and 11, 103, 1912. Howe and Hawk, *Jour. Am. Chem. Soc.*, 33, 215, 1911; *Am. Jour. of Physiology*, 29, xiv., 1912, and 30, 174, 1912.

[2] We take this opportunity to acknowledge the material assistance which we received from the department of chemistry of the University of Illinois in defraying the expenses of the research.

fibers seem widely separated from each other, giving the entire muscle a very loose appearance. In cross section they are seen to consist of a dense central portion, which stains more or less strongly, surrounded by a colorless material. There is, however, no indication of a swelling of the muscle fibers as there is likewise no evidence of fatty degeneration, but they apparently undergo a process of liquefaction similar to that described by Miescher in the Rhein Salmon occasioned also by protracted fasting while it remains in fresh water. The nuclei are extremely irregular in outline and stain faintly.

In the voluntary, or striated muscle fibers, the cross markings lack the usual distinctness. Swelling or granular degeneration, such as described by Statkewitch, was never seen in our material.

Of all the organs of the body the liver is taxed most heavily during inanition inasmuch as it must take care of the products of metabolic activity of all other organs besides sustaining itself. It is natural to expect, therefore, to find the changes in the structure of the liver cells of a most pronounced character. Indeed, in the material under our examination a variety of degenerative phenomena has been observed. Considering the great difference in the degree of degeneration of the liver from animals which have all died of starvation it follows that death is not necessarily preceded by extreme cellular transformation. In our material every gradation from very slight changes to complete fatty degeneration of the liver cells could be observed. In two dogs which fasted 30 and 48 days respectively, whereby they lost 46 and 53 per cent. of their weight, there has been very little fatty degeneration in the liver. Some cells, however, were coarsely granular and others were riddled with vacuoles. In the case of the fox, which in 13 days of absolute fasting lost only 13 per cent. of its weight, the cells were found to be hollowed out by vacuoles of various sizes. These frequently encroach upon the nucleus and distort its shape as may be seen in Fig. 1. The vacuoles never show a very sharp outline, their boundary being more commonly diffuse and indefinite. In one extreme case of degeneration the liver presented complete transformation of its cells into typical fat cells. The polygonal shape of the cells was retained but the protoplasm was reduced to a mere band enclosing a mass of fat. The cells seemed rather distended. The nuclei, pushed out to the periphery and usually into a recess of a corner, were flattened against the wall. Their staining capacity as well as that of the protoplasm was very feeble. The fatty degeneration was not equally intense in every portion of the liver, and here and there groups of intact liver cells could be seen whose poor staining power was the only evidence of degeneration.

No particular changes have been observed in nuclei. Cells with more than one nucleus are not uncommon, but these are found also in the normal liver. Phenomena of chromatolysis and vacuolization of the nuclei described by Statkewitch were never observed by us.

The histological structure of the stomach and intestine shows no striking changes. In sections of the stomach the oxyntic or parietal cells of the fundus glands are most conspicuous owing to their relatively large size and deep staining capacity. Their protoplasm is very granular. The other cells of the gland are small and their protoplasm is thin and practically colorless. The nuclei are usually normal, but in some portions, especially near the proximal end of the gland, they are much elongated and pressed against the cell wall adjoining the basal membrane. The two figures in the plate, 2 and 3, one a cross section of the upper region of the gland, the other a longitudinal section through the base of the gland, show these points. The clear, transparent character of the protoplasm is very well seen in the former, Fig. 2. The nuclei are always near the basal membrane.

The points brought out in the study of the fundus glands are also essential for all other glands as well as the mucous membrane of the intestine. The cells stain very feebly, their protoplasm being free of any granules. The nuclei migrate toward the basal membrane.

The phenomenon of particular interest, especially when viewed in the light of certain results of bacteriological studies on the permeability of the intestinal canal, is the invasion of the tissue underlying the mucous membrane as well as of the cells of the mucous membrane itself by numerous leucocytes. These occur not only singly but in groups of several cells together and occasionally accumulate in masses resembling solitary glands.

The submaxillary gland presents a few changes which are worth pointing out. The protoplasm of its cells, as was seen also in other gland cells, is thin in character and fails to take up the stain. Many cells are without nuclei, and the darkly stained crescent cells though present are generally flattened out and cap the outside of the alveoli like a narrow band. Fig. 4 shows that the submaxillary gland at the fatal termination of a protracted fast has all the appearance of a resting gland.

The most interesting set of modifications is to be observed in the kidneys. There one encounters various forms of degeneration and their distribution in the kidney is quite significant. We have already mentioned in discussing the changes in the liver that the extent of the degeneration of the histological elements apparently bears no relation to the death of the fasting animal as one of its direct causes. This statement holds equally true for the kidneys, where we found likewise a very wide range of modifications at the time of the animal's death.

The glomerulus has the usual lobulated structure but the Bowman capsule enclosing it is invariably thickened as in the case of nephritic kidneys. The cells of the convoluted tubules have a coarsely granular content and are invariably vacuolated. In some instances the vacuolization is so extensive as to give the tubule a striking honeycombed appearance. In Fig. 5 which is from a section of the kidney of the fox, this is shown very clearly. Similarly Fig. 6, which represents a section of a tubule in the kidney of a fasting dog, shows extensive vacuolization and the absence of boundaries between the cells. This last phenomenon, namely, the formation of a syncitium is characteristic not only of the kidney but also of the liver where the cells seem to melt together. In the ascending and descending limb of Henle's loop, however, vacuolization is a very rare occurrence, particularly in the later. The tubules were generally very granular in structure and contained frequently casts of various kinds, cellular, hyaline, etc. Fig. 5 is interesting furthermore on account of the well-preserved ciliated band lining the lumen of the tubule. The nuclei of the tubules are small and more or less irregular in shape. The cells of the collecting tubules show hardly any effect. The protoplasmic content is very clear and free of granules. Here and there cells are found which have no nuclei. But when present the nuclei are relatively large and round, frequently bulging out into the lumen owing to the diminution of the cubical cells.

Before concluding this description of the changes which were observed in the tissues of fasting animals, a few words may be said concerning the condition of the testes and ovaries. In the former we failed to find any dividing cells. The nuclei were of the characteristic large round shape, whose chromatic content was intensely stained. It is noteworthy that in a very large proportion of the tubules the chromatic substance of all the nuclei was massed together to one side, as in the case of synizesis. It is hardly possible that this should be due to an artifact, as the nuclear condition varied in different tubules, but the former was found in most of them. The ovary which was examined seemed normal in every respect with numerous eggs in all stages of growth. We examined a number of fully developed eggs which were perfectly normal in every detail of their structure.

Bearing in mind that in inanition the organism is obliged to draw upon its own resources to derive the energy necessary for its maintenance, the metaplasmic material stored up in its cells and the depôts of fat are first to yield their quota to this stringent need. With the prolongation of the fast, as these reserve materials become reduced in quantity and at last disappear altogether, the substance of the cell body proper must contribute to the organism's demand for nourishment. It is now a well-established fact that various organs and tissues share unevenly in the support of the starving organism. As would be expected *a priori*, those elements of the organism the integrity of which is indispensable to its continued existence resist the pressure of the unfavorable conditions longest. This is true not only for the different systems of organs, but also for the minutest element of the organism, the cell, where the nucleus is usually the last part to fall prey to the exhausting effect of the fast. The nervous system likewise maintains its weight practically at a constant level as well as it preserves its morphological integrity until a very advanced stage in the fast.

Degenerative changes do not, as a rule, occur in any of the tissues so long as the reserves of the body have not yet been entirely exhausted. The early appearance of fat globules in the liver of fasting animals led Mottram to believe that this process must be a physiological and not a pathological one. A similar opinion was likewise expressed earlier by Gilbert et Jomier. Mottram showed by means of histological examination of the liver of rabbits and guinea-pigs as well as by actual chemical investigation that with the advance of the fast an infiltration of the liver cells with fat from the depôts does take place. None of the authors who gave attention to this matter studied the liver of animals in very advanced stages of a fast. In our own case the animals succumbed after a loss of about 50 per cent. of their weight. There was very little histological evidence of a fat accumulation, but vacuolization of the cells was most prominent. It is hardly conceivable that the vacuoles were produced by the removal of the fat content by the reagents used in preservation in as much as it has been shown above that in one instance a liver was observed the cells of which in certain localities have undergone complete fatty degeneration. We are aware of the fact, of course, that a parallelism does not exist between histologically and chemically demonstrable fat in tissues. It may be that the infiltration of the liver with fat, especially in the early period of fasting, which is now proven beyond reasonable doubt (Mottram, Smirnow) and is very properly considered a physiological phenomenon, is concerned with the transfer of depôt fat to the rest of the tissues as food, while fatty degeneration of the liver cells, such as we observed over certain areas, is an independent phenomenon and is accompanied by the loss of the normal functional power of the cells. The view expressed here that the infiltration of the liver with fat may have to do with the conveying of the fat as nutriment to the starving tissues is borne out by Mottram's interesting observation on the qualitative change of the liver fat on different days of a short fast. There is a striking parallelism between the pure fatty acids present in the liver and the fat-quotient, *i. e.*, the ratio of the total fat of the liver to the initial body weight, showing that whenever an increase in fat content, *i. e.*, an infiltration, occurs it is due to an accumulation of fatty acids. If this view of the rôle of the liver is correct it may also clear up the problem of the cause of the premortal rise in the nitrogen elimination. The latter was thought to be due either to an exhaustion of the entire supply of fat or to an excessive disintegration of cells. Neither the one nor the other of these hypotheses can be considered beyond criticism, because even in animals succumbing to a much protracted fast there is still sufficient fat present,[1] whereas there is no histological evidence of an unusual cellular destruction towards the end of the fast. A morphological and physiological degeneration of the liver interfering in some manner unknown to us with the endogenous fat metabolism probably results in an increased demand upon the body proteins which hastens the death of the animal.

In this connection it is interesting to point out that fat has never been demonstrated in tissues which in the fasting organism are among the strongest consumers, such as nervous and muscular tissues. The glandular tissues on the other hand, which are more or less deprived of their proper activity during a fast invariably show the presence of fat globules, according to Nicolaides. Later these fat globules disappear, leaving "empty spaces" which evidently correspond to our vacuoles. Nicolaides observed that in the gland cells of the duodenum and the pylorus small fat globules appear as soon as the animals commence to fast, whereby they invariably assume a regular arrangement in two parallel rows. We cannot agree with Nicolaides who considers the fat globules as "degenerative," and certainly see no reason for his assumption,—since their arrangement within the cell points against the supposition of a migration from fat depôts,—that they are formed from the protein constituents. The fact that in the submaxillary gland the fat globules appear only in the albuminous cells but never in the mucous or crescent cells, which has been observed by both Statkewitch and Nicolaides, cannot be taken as good proof of a formation of fat from protein. Also these facts become plain in the light of our hypothesis that the early appearance of the fat globules speaks decisively against any supposition that they result from degenerative transformation. We believe, on the contrary, that this is simply due to a qualitative transformation of the fat present in the cells whereby it becomes histologically visible. Since the staining reagents which are used in demonstrating the presence of fat globules are such that they react only with unsaturated fats, the appearance of the globules indicates that with the beginning of the fast a process of desaturation and probably the formation of fatty acids is started preliminary to the absorption of this fat to serve the nutritional needs of the organism.

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[1] In the case of a dog which fasted 117 days and subsequently 104 days there were large masses of fatty tissue in the abdominal cavity at the time of death.

The results of studies on fasting unicellular organisms where the conditions are simpler and easier to be appreciated support the view that vacuolization is one of the earliest and the most common degenerative process which ensues with the exhaustion of the reserves of the cell. Wallengren indeed in his most valuable research on inanition of infusoria distinguishes two periods, before and after the reserve material is exhausted. The former is accompanied by a gradual diminution of the animal, while in the second period the endoplasm becomes honey-combed with vacuoles of various sizes.

Vacuolization has been observed in various tissues: in ganglionic cells of the heart (Statkewitch), in the cells of the motor ganglia of the anterior horn (Schäffer) in bone marrow (Soltz), in the nephridial epithelium, etc.

Before concluding the paper mention should be made of another degenerative process which develops in the course of inanition. We refer to the gradual melting away of the cell boundaries which, with their complete disappearance, may even result in the formation of a syncytium. One of us described this condition in the liver of fasting salamanders. We also observed this phenomenon in our liver and kidney preparations. Similar observations have been made in fasting lower animals (Schultz). In the study of the salamanders it was shown how rapidly the cell walls are built up again around the intact nuclei as soon as the emaciated animals are once more given food.

At last the more or less universal loss of staining capacity due to the degenerative transformation produced by inanition and described by practically all authors must be pointed out.

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EXPLANATION OF FIGURES.

All figures have been made with the camera lucida and under the same magnification, using an objective No. 6 with an ocular piece No. 4 at normal tube length.

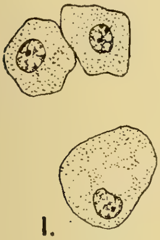
FIG. 1. A group of liver cells of a fasting fox.

FIGS. 2 AND 3. Cross-section and longitudinal section of fundus gland of a fasting dog.

FIG. 4. Section of submaxillary gland of fasting dog.

FIG. 5. Section of tubules in kidney of fasting fox.

FIGS. 6 AND 7. Section of convoluted tubule and of Henle's loop in kidney of fasting dog.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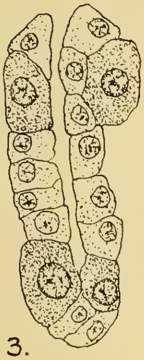
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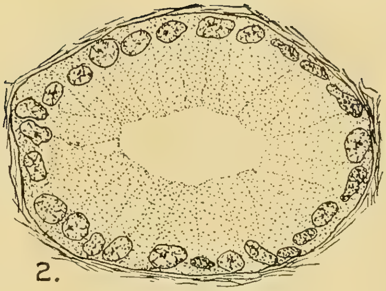
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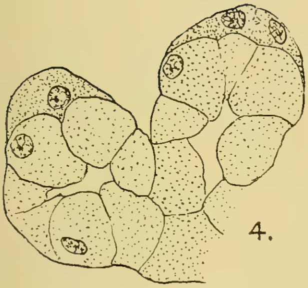
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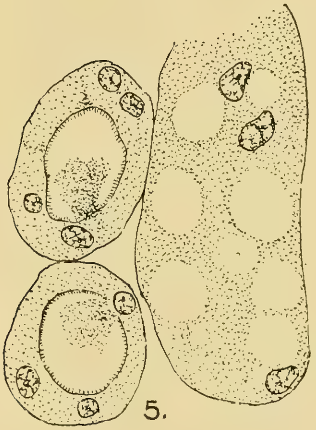
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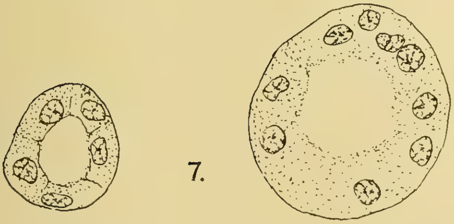
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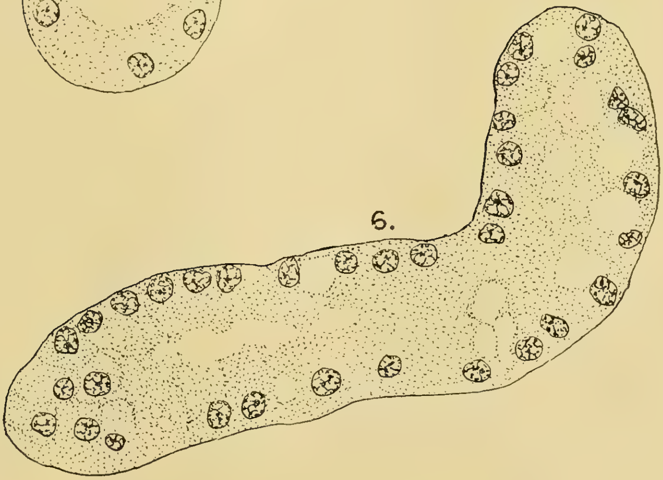
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MORGULIS, HOWE AND HAWK.

THE OLFACTORY SENSE OF COLEOPTERA

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INTRODUCTION AND METHODS.

In the investigation here recorded two objects have been kept in view: (1) To make a careful study of the morphology and physiology of the olfactory pores of beetles, and (2) to determine experimentally whether or not the olfactory organs lie in the antennæ.

Since those investigators, who have performed experiments on beetles with mutilated antennæ, have failed to study sufficiently the behavior of the insects investigated, the responses observed have misled them in determining the seat of the olfactory organs. Entomologists are generally agreed that the organs of smell in beetles lie in the antennæ, but when the results of those who have performed experiments on beetles are carefully considered, it is seen that some beetles with amputated antennæ smell practically as well as unmutilated ones, while other beetles are materially affected when the antennæ are mutilated. Hicks (1857 and 1860) discovered some peculiar organs (called olfactory pores by the present writer) on the wings and legs of beetles and he suggested that they have an olfactory function. Lehr[1] discovered the same organs on the peduncles of the elytra of *Dytiscus marginalis*. The present writer ('14*a* and *b*) made a comprehensive study of the olfactory pores in Hymenoptera and he ('14*c*) gives a complete review of the literature pertaining to the sense of smell in insects. The present paper embodies the results of a careful study of the olfactory pores in Coleoptera in much the same manner as pursued on those in Hymenoptera.

To obtain material for the study of the disposition of the olfactory pores, adult specimens were used. In regard to preparing the specimens with caustic potash and to bleaching them with chlorine gas, the reader is referred to the writer's work on Hymenoptera ('14*b*, p. 295).

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[1] Lehr's paper, which deals only with the morphology of these organs, was overlooked until after my paper had been sent to press. Lehr has not seen any of my papers on this subject because my first one ('14*a*) appeared only three months before his, and my second one ('14*b*) appeared in the same month as his.

To obtain material for the study of the internal anatomy of the organs herein discussed, beetles just emerging from the last pupal stage were mostly used. At this stage the chitin is soft, the wings are usually expanded, and the sense organs are fully developed. In order that the desired stages of beetles might be had, many larvæ and pupæ of various Coleoptera were collected on plants and in rotten stumps and logs. These immature insects were reared in the laboratory. When each one of them had reached the proper stage, it was killed and parts of it were put into a fixing fluid.

The writer ('14*a*, p. 268) describes the usual method of embedding with celloidin and paraffin. Since then, a rapid method has been used which is described in detail as follows: The various appendages of the insects are removed, and are cut into small pieces, which are immediately dropped into a modification of Carnoy's fixing fluid. This fluid, containing equal parts of absolute alcohol, chloroform, and glacial acetic acid, with corrosive sublimate to excess, should be kept in a glass-stoppered bottle so that it may not lose its fixing ability by air being mixed with it. Also, while dropping material into vials containing this fluid, the stoppers of the vials should not be removed longer than absolutely necessary. When the material sinks to the bottom of the vial, it is removed and is thoroughly washed in 85 per cent. alcohol. It is then preserved in 85 per cent. alcohol. When ready for embedding, the material is cut into pieces from two to four millimeters in length. These pieces are then put into 95 per cent. alcohol containing eosin. When sufficiently stained, they are placed in a vial containing absolute alcohol and cedar oil. As soon as they sink through the alcohol into the oil and lie on the bottom of the vial, the alcohol and oil are removed. A small amount of ether is then poured into the vial. Five minutes later the ether is removed, and thin celloidin is poured into the vial. Ten minutes still later the thin celloidin is exchanged for thick celloidin. After remaining in the thick celloidin five minutes, the pieces of material are removed and are put into a vial of chloroform where they remain five minutes. They are then embedded in 55° M.P. paraffin for five minutes. The sections were cut from five to ten microns in thickness and when they failed to ribbon the microtome knife was warmed. From this stage on the sections are treated like ordinary paraffin sections with the following exceptions. A rather thick film of fresh Mayer's albumen is spread upon each slide. After drawing the water from the slide upon which are mounted the sections, the latter are flattened to the slide by using a piece of wet tissue paper. No heat is used for straightening the ribbons on the slides because the least amount of heat blisters the celloidin. After drying over night, most of the sections adhere to the slides while being passed through the reagents, but to be sure of not losing any sections, the slides were sometimes wrapped in tissue paper and thread was then firmly wound around the paper. Instead of using absolute alcohol a mixture of equal parts of absolute alcohol and chloroform is employed so that the celloidin may not be dissolved, and instead of using eosin in 95 per cent. alcohol as a counter stain, the eosin is put into a mixture of the absolute alcohol and chloroform. The sections were stained in Ehrlich's hematoxylin from 10 to 15 minutes, the time depending on their thickness and whether or not they were wrapped in tissue paper.

The writer is grateful to Mr. H. S. Barber of the Bureau of Entomology for most of the dried specimens used which belonged to the collections of the U. S. National Museum. Mr. Barber is also to be thanked for the identification of all the beetles used in the experimental part of this work.

MORPHOLOGY OF THE OLFACTORY PORES.

Before experimenting to determine the function of the organs called the olfactory pores by the writer ('14*a*), the distribution and number of these pores in many beetles were studied.

Disposition.

In making a comparative study of the disposition of the olfactory pores in beetles, 50 species, belonging to 47 genera and representing 34 families, were used. With the exception of two species used for individual and sexual variations, only one specimen of each species was studied. Whenever a portion of an appendage or an entire appendage was missing or was badly mutilated in being prepared for study, the number of pores on this portion or entire appendage was regarded the same as the number found on the corresponding portion or entire appendage on the opposite side of the body. Since the pores on only one specimen for each species were counted, the total number of pores recorded can not be a fair average. Besides this error, there is also a probable error of not less than 10 per cent. on an average for all the specimens. In the smaller specimens the probable error is perhaps not more than two or three per cent., but in some of the larger ones, this error is probably more than 10 per cent. The pores on only the legs, elytra and wings are included in the total numbers. Other parts of the insects were not examined, and it is quite possible that olfactory pores may be found on some of the parts not examined, particularly on the mouth parts.

(*a*) *Epilachna borealis.*

Since the lady beetle, *Epilachna borealis*, is most conveniently studied and as its pores are typical for most of the smaller beetles, the disposition of its pores will be described in detail, and then the variations found in the other species will be given.

The elytra and wings have dorsal and ventral surfaces, and the legs may be divided for description into two surfaces. The inner surface faces the body of the beetle and the outer surface is directed from the body. On the specimen examined, one group of pores was found on the peduncle of each elytron; three groups besides a few scattered pores on each wing; and two groups besides a few scattered pores on each leg. The groups and scattered pores are located as follows: Group No. 1 lies on the dorsal surface of the peduncle of the elytron with its distal or broader end against or just beneath the basal margin of the elytron (Text-fig. 1*A*, *BM*). Under a high-power lens, it is seen that this group lies on the radial plate (Text-fig. 1*B*, *RP*) between the muscle disk (*MD*) and the subcostal head (*ScH*). The distal ends of these heavy chitinous plates sometimes lie beneath the basal margin (*BM*) of the elytron so that all or a portion of the group may be concealed. In such a case it is necessary to pull the peduncle from beneath the base of the elytron in order to count the pores. Group No. 1 on the left elytron consists of 71 pores (Plate I., Fig. 4), while the same group on the right elytron has 78 pores.

Groups Nos. 2, 3 and 4 lie on the dorsal surface of the wing on the radius (Text-fig. 1*C*, *R*). No. 2 lies on the extreme anterior end of the radius and it is usually difficult to count its pores, because the surface of the radius at this place is greatly arched causing some of the pores to lie on the top of the arch while the remainder of them lie on the side of the arch facing the anterior margin of the wing. Nos. 3 and 4 are found on the radius where the media (*Me*) joins the radius. On the right wing, No. 2

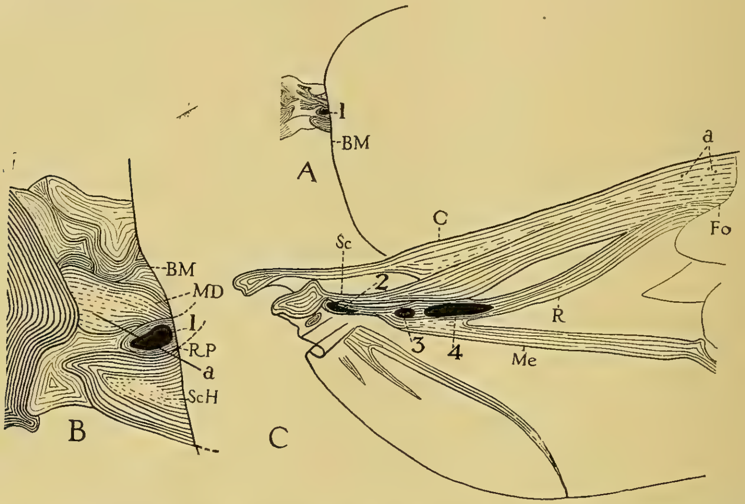

FIG. 1. Portion of left elytron and left wing of the lady beetle, *Epilachna borealis*, showing groups 1 to 4 of olfactory pores, as indicated by the numbers 1 to 4; *A* shows relative sizes of peduncle of elytron and group of pores on peduncle when compared with size of basal margin (*BM*) of elytron; *A* and *B*, dorsal surface of peduncle of elytron, showing position of group 1 of olfactory pores on radial plate (*RP*) between muscle disk (*MD*) and subcostal head (*ScH*). The lower side of each drawing is the outer margin of the elytron. *A*, × 8; *B*, × 45; *C*, dorsal surface of wing, showing position of groups 2 to 4 of olfactory pores on radius (*R*), × 8; *a*, position of scattered pores on ventral side of wing on union of costa (*C*) and subcosta (*Sc*) near fold of wing (*Fo*). Sometimes a group is found on the media (*Me*) just below group 4.

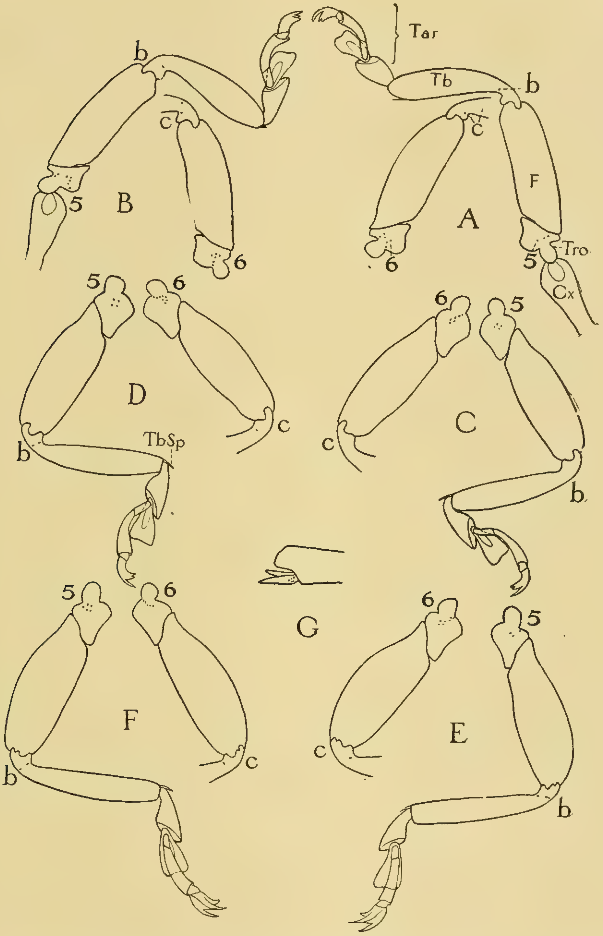

FIG. 2. Position of olfactory pores on legs of beetles, × 8; *A–F*, legs of lady beetle, *Epilachna borealis*, showing position of groups 5, 6, *b* and *c* of olfactory pores. The drawing of each leg in which the tarsus (*Tar*) is shown represents the outer surface of that leg, and the drawing not showing the tarsus represents the inner surface of the same leg. *A*, right front leg; *B*, left front leg; *C*, right middle leg; *D*, left middle leg; *E*, right hind leg; *F*, left hind leg. *G*, distal end of tibia from front leg of *Epicauta marginata*, showing five olfactory pores on one of the two tibial spines.

consists of 55 pores; No. 3 of 43 pores; and No. 4 of 43 pores. On the left wing, No. 2 consists of 50 pores; No. 3 of 43 pores; and No. 4 of 46 pores.

Group *a* of the scattered pores lies on the ventral surface of the wing near the anterior margin of the wing a short distance from the place where the wing folds (Text-fig. 1*C*, *a*). On the right wing it consists of three pores and on the left wing of five pores.

Groups Nos. 5 and 6 are located at the proximal end of the trochanter (Text-fig. 2*A–F*, *Tro*), No. 5 lying on the outer surface and No. 6 on the inner surface. No. 5 usually extends only about half way across the leg, while No. 6 extends nearly all the distance across the leg. No. 5 on each leg consists of five pores, except on the left front leg where there are seven in it. On the right side No. 6 on each leg consists of seven pores, whereas on the left side on each of the middle and hind legs it consists of eight pores but of only six on the front leg.

Groups *b* and *c* of the scattered pores lie at the proximal end of the tibia (Text-fig. 2*A–F*, *Tb*), group *b* being located on the outer surface and group *c* on the inner surface. Group *b* on each front leg consists of only one pore; on the right middle leg it has one pore, but on the left middle leg it has two pores; on the right hind leg it has three pores while on the left hind leg it consists of two pores. Group *c* on each front leg has three pores, whereas on each of the middle and hind legs it has only one pore.

All six legs of the specimen of *Epilachna borealis* examined bear 95 olfactory pores; both elytra carry 149 pores, and both wings carry 288 pores. All of these combined make 532 olfactory pores.

(*b*) *Other Species.*

The greatest variation found in the olfactory pores of the other species examined is in regard to the total numbers of the pores. The second greatest variation is in regard to the distribution of the pores on the wings. This variation and other minor ones will now be given and a discussion of the total numbers of the pores will be presented last. For sake of brevity, instead of using the long scientific names of the beetles, the species will be numbered from 1 to 50, and those interested in associating the names of the species with the variations described may do so by referring to the names and numbers of the species in the table on page 419.

A group of pores (No. 1) was found on the peduncle of each elytron. This group in 46 species is definite, that is, the pores are close together and are not scattered as they are in the other four species (Nos. 2, 34, 35, 44). In some beetles it is almost impossible to identify the various chitinous plates in the peduncles of the elytra, but as far as can be ascertained the definite groups of pores are located on the radial plates, while the scattered groups may spread over two or more of the plates. In shape these groups are round, oblong and triangular. The triangular-shaped ones are most common. As a rule, the more pores in this group, the smaller they are and the closer they are together. In three species (Nos. 23, 29, 36) the pores in this group are comparatively large, while those in the lady beetles are medium in size. *Osmus* with 12 pores on both elytra has the least number and *Hydrophilus* with 310 pores on both elytra has the largest number. In regard to the total numbers of pores on the elytra for the 50 species, the reader is referred to the table on page 419.

The three beetles, *Osmus*, *Clinidium* and *Cysteodemus*, are wingless. No rudiments of the wings were even found. The number of groups of pores on each wing of the other species varies from 1 to 4. Ten species (Nos. 1, 10, 13, 14, 16, 17, 18, 21, 22, 47) have only one group on each wing. One wing of *Lucidota* has one group while the other wing has two groups. Twenty-one species (Nos. 4, 5, 8, 11, 20, 24, 25, 26, 28, 30, 31, 32, 36, 38, 40, 42, 43, 45, 46, 49, 50) have two groups on each wing. Twelve species (Nos. 3, 6, 7, 9, 15, 23, 29, 33, 37, 39, 41, 48) have three groups on each wing. Three species (Nos. 12, 34, 35) have four groups on each wing. When only one group is present on each wing it usually occupies the position of Nos. 3 and 4 of *Epilachna borealis* on the radius (Text-fig. 1*C*). It may be no longer than No. 4 of *Epilachna*, or it may extend nearly all the distance to the fold of the wing (*Fo*). When two groups are present on each wing, one is similar to No. 2 of *Epilachna* and the other is similar to Nos. 3 and 4 united. The latter group may or may not extend all the way to the fold of the wing. In *Collops*, 20 pores were found on the ventral side of one wing besides the two groups on the dorsal surface. When three groups are found on each wing, they may be located like those of *Epilachna*, or two of them may lie on the radius and the third one on the media. The largest one is similar to No. 4 of *Epilachna* and it may or may not extend all the way to the fold of the wing. When the third group lies on the media, as in *Orthosoma* (Plate II., Fig. 31), it occupies a position just beneath the larger group on the radius. Its pores are generally scattered considerably. When four groups are found on each wing, one of them lies on the subcosta, two on the radius and one on the media. It is common for the distal end of the largest group on the radius of any wing to become attenuated so that a row of pores may extend nearly, if not all, the way to the fold of the wing. The farther this row of pores extends along the radius the farther apart are the pores. It is also common for the largest group on the radius to consist of pores of two sizes. The diameters of the larger pores may be two or three times those of the smaller ones. The larger pores extend lengthwise through the center of the group. Eight species (Nos. 1, 3, 4, 5, 6, 33, 36, 38) have pores as just described. The pores in this group of seven other species (Nos. 10 to 14, 23, 30) are also of two sizes, but there is not such a great difference in the sizes of the smaller and larger pores, as in the pores of the preceding eight species. These pores are also comparatively larger. All the pores on the wings of nine species (Nos. 7, 9, 16, 17, 18, 20, 22, 31, 40) are of about the same size and they are comparatively large. All the pores on the wings of the remaining species are of about the same size, but they are comparatively small. *Coxelus*, the smallest beetle examined, with 130 pores on both wings has the least number, while *Orthosoma*, perhaps the largest beetle examined, with 982 pores on both wings has the greatest number.

The trochanters never fail to possess at least a few pores. The trochanter with the fewest pores has two, whereas the one with the most has 59. As a rule, the more pores on a trochanter, the smaller they are. The pores are generally located at the proximal end of this segment in about the same arrangement as represented in *Epilachna* (Text-fig. 2*A*–*F*), but occasionally they are considerably scattered, and a few may be found at the distal end of the segment.

A pore was found at the proximal end of one or more femurs belonging to each of 18 species (Nos. 1, 2, 4, 7, 8, 10, 11, 13, 16, 17, 18, 20, 21, 22, 24, 31, 48, 49), and from one to three pores were found at the proximal end of each femur of *Elater*.

While it is common to find one or more pores at the proximal end of a tibia, many of these segments are entirely devoid of olfactory pores. The greatest number of pores found on any tibia at this place is nine. In each of the tibio-tarsal articulations of the front and middle legs belonging to *Cotinis* from 7 to 11 pores were found. Pores were found in the tibial spines (Text-fig. 2*G* and Plate II., Fig. 27) of 15 species (Nos. 9 to 11, 20 to 25, 31, 32, 34, 35, 45, 48). The pores usually lie on the bases of the large spines. The largest number of pores found on a single tibial spine is 12. Of the 50 species examined, *Passalus* has the most pores on these spines.

Pores were found on the tarsi of 13 species (Nos. 1, 2, 4, 10 to 12, 16, 18 to 21, 25, 31). The greatest number found on a single tarsus was 37. *Osmus*, one of the three apterous species, has the most pores on its tarsi.

Eleven species (Nos. 6, 26, 27, 29, 31, 32, 33, 38, 42, 44, 47) were found with no pores on the legs except those on the trochanters and on the tibial spines. *Cybister* with 49 pores on all six legs has the least number on these appendages, while *Podabrus* with 341 pores on all six legs has the largest number.

No special examination was made to find any structure other than the olfactory pores, nevertheless, minute pores were seen in 15 species (Nos. 7, 8, 14, 15, 16, 20, 25, 27, 29, 36, 40, 44, 45, 46, 48). These pores were seen on various parts of the beetles, but particularly on the legs and elytra. They usually lie near the bases of the hairs, but sometimes they lie a considerable distance from the hairs. Since they are many times smaller than the olfactory pores, without exception they are probably the pores belonging to hypodermal glands, as will be shown for those of *Epilachna* on page 423. However, a careful comparative study of these pores is needed before anything definite can be said about them.

Coxelus, the smallest species examined, has a total number of 273 pores which is the smallest number of all the winged species, while *Orthosoma*, perhaps the largest species examined, has a total number of 1,268 pores, which is the largest number of all the species examined. As a rule the smaller the species, the larger are the pores, comparatively speaking, and the fewer they are. Likewise, the larger the species, the greater is the number of its pores and the smaller they are. As a rule there are no generic and specific differences, except variations in number of pores, the amount of variation depending on the size of the individuals compared. Judging from the sizes of the four water beetles examined, the pores on their legs are fewer and smaller than those on the legs of any other beetle examined. Pores were found only on the trochanters of *Cybister*, while a few were also seen on the femurs and tibiæ of the other three water beetles. The number of pores on the legs of these beetles are as follows: *Cybister*—49, *Dineutes discolor*—65, *Dineutes vittatus*—98, and *Hydrophilus*—93. These numbers indicate that the better the legs are adapted for locomotion in water, the fewer pores they have.

The small total numbers of pores of *Osmus*, *Clinidium* and *Cysteodemus* are due to the absence of wings. In *Osmus* and *Clinidium* more pores are found on the legs than might be suspected. The tarsi of *Osmus* have more than the tarsi of any other beetle while the tarsi of *Clinidium* have more than suspected.

The following table (p. 419) includes the family, name and number, the olfactory pores on the legs, elytra, wings, and the total number of pores of each of the 50 species examined. In the preceding pages the beetles are usually referred to in this table by their respective numbers.

(*c*) *Individual and Sexual Variations.*

For this study five males and five females each of *Harpalus pennsylvanica* and *Leptinotarsa 10-lineata* were used. No individual and sexual variations were found, except slight variations in the number of pores. The total numbers of pores of the males of *Harpalus* vary from 550 to 580 with 570 as an average; those of the females of *Harpalus* from 575 to 699 with 628 as an average. The average number of pores for males and females of *Harpalus* is 599. The total numbers of pores of males of *Leptinotarsa* vary from 665 to 780 with 722 as an average; those of the females

TABLE I.

THE NUMBER OF OLFACTORY PORES ON THE LEGS, ELYTRA AND WINGS OF COLEOPTERA.

Family.	Name and Number of Species.	No. of Pores on Legs.	No. of Pores on Elytra.	No. of Pores on Wings.	Total No. of Pores.
Cicindelidæ.....	1. *Cicindela vulgaris*	180	29	924	1,133
	2. *Osmus* sp.	290	12		302
Carabidæ.......	3. *Calosoma scrutator*	140	62	869	1,071
	4. *Harpalus caliginosus*	180	69	600	849
	5. *Harpalus pennsylvanica*	107	39	453	599
Dytiscidæ.......	6. *Cybister fimbriolatus*	49	180	917	1,046
Gyrinidæ.......	7. *Dineutes discolor*	65	46	435	546
	8. *Dineutes vittatus*	98	42	532	672
Hydrophilidæ....	9. *Hydrophilus triangularis*	93	310	662	1,065
Silphidæ........	10. *Necrophorus marginatus*	111	60	652	823
	11. *Silpha inæqualis*	118	30	664	812
Staphylinidæ ...	12. *Staphylinus macrulosus*	110	23	493	626
Scaphidiidæ.....	13. *Scaphidium quadguttatum*	111	96	185	392
Coccinellidæ.....	14. *Coccinella 9-notata*	93	132	310	535
	15. *Epilachna borealis*	95	149	288	532
Endomychidæ...	16. *Endomychus biguttatus*	144	31	178	353
Erotylidæ......	17. *Megalodacne heros*	102	120	383	605
Colydiidæ......	18. *Coxelus guttulatus*	93	50	130	273
Rhyssodidæ	19. *Clinidium sculptile*	131	40		171
Cucujidæ.......	20. *Cucujus clavipes*	107	104	325	536
Mycetophagidæ .	21. *Mycetophagus punctatus*	165	135	379	679
Dermestidæ.....	22. *Dermestes marmorata*	90	80	570	740
Histeridæ.......	23. *Hister depurator*	74	80	249	403
Trogositidæ.....	24. *Tenebroides castenea*	96	113	296	505
Elateridæ.......	25. *Elater apicatus*	111	130	365	606
Buprestidæ	26. *Melanophila longipes*	186	110	278	574
Lampyridæ.....	27. *Lucidota californica*	177	123	257	557
Telephoridæ.....	28. *Chaulcognathus pennsylvanica*	308	157	445	910
	29. *Podabrus comes*	341	157	280	778
Malachidæ......	30. *Collops bipunctatus*	120	101	248	469
Lucanidæ.......	31. *Platycerus quercus*	158	160	536	854
	32. *Passalus cornutus*	203	184	782	1,169
Scarabæidæ.....	33. *Canthon lævis*	62	180	724	966
	34. *Cotinis nitida*	162	39	934	1,135
	35. *Euphoria sepulchralis*	90	36	613	739
	36. *Osmoderma scabra*	127	182	782	1,091
Cerambycidæ....	37. *Orthosoma brunneum*	79	207	982	1,268
	38. *Callimoxys fuscipennis*	87	175	510	772
	39. *Cyllene robiniæ*	110	40	629	779
Chrysomelidæ...	40. *Leptinotarsa 10-lineata*	115	130	476	721
Bruchidæ.......	41. *Bruchus pisi*	65	137	352	554
Tenebrionidæ....	42. *Tenebrio molitor*	66	145	462	673
	43. *Uloma impressa*	80	119	361	560
Meloidæ........	44. *Cysteodemus armatus*	133	39		172
	45. *Epicauta marginata*	157	94	504	755
	46. *Epicauta pennsylvanica*	125	100	440	665
Rhipiphoridæ....	47. *Myodites scaber*	154	260	350	764
Rhynchophora:					
Rhynchitidæ. .	48. *Rhynchites bicolor*	84	148	480	712
Otiorhynchidæ	49. *Cephus latus*	71	111	382	564
Curculionidæ .	50. *Zygops seminivius*	65	116	392	573
Variation		49–341	12–310	130–982	273–1,268[1]

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[1] The total number of pores of apterous species are not included.

of the same species from 661 to 785 with 720 as an average. It is thus seen that the females of *Harpalus* have a few more pores than the males, while the males and females of *Leptinotarsa* have the same number of pores.

STRUCTURE.

In the preceding pages it has been shown that most of the variations in regard to the disposition of the olfactory pores are slight. In the following pages it will be shown whether or not this is true for the structure of these pores.

(*a*) *External Structure.*

When examined under a low-power lens, the olfactory pores may be easily mistaken for hair sockets from which the hairs have been removed. When more carefully observed under a high-power lens, a striking difference in external form is usually seen, but sometimes it is difficult to distinguish the pores from hair sockets. The pores appear as small bright spots when a strong transmitted light is used. Each bright spot has a dark boundary or pore wall (Plate I., Fig. 1, *PorW*).[1] Near the center of this boundary is a transparent spot, the pore aperture, which may be round, oblong, slit-shaped, or club-shaped. On the legs the pore apertures may be round (Fig. 2, *PorAp*), oblong (Fig. 3, *PorAp*), slit-shaped or club-shaped (Fig. 1, *PorAp*). On the elytra and wings they may be round or oblong (Figs. 4 to 8). The hair sockets (Figs. 1 and 2, *PorWHr*) are generally smaller than the olfactory pores and the pores of the hypodermal glands (Figs. 1 and 2, *PorWGl*) are easily distinguished from the hair sockets and olfactory pores by their small size.

(*b*) *Internal Structure.*

All the olfactory pores studied are more or less flask-shaped structures. They are of three general types. In the most common type, as found in *Uloma*, the mouth of the pore (Figs. 9–12, *Mo*) is flaring and the sense cell (Fig. 12, *CS*) lies in the lumen of the appendage outside the pore cavity. The chitinous cone (Fig. 9, *Con*) never occupies more than one fourth of the pore cavity and usually much less (Fig. 12, *Con*). The cone always stains less deeply than the surrounding chitin, and it is common to see a hypodermal secretion (Figs. 9 and 10, *HypS*) inside the pore cavity. The sense fiber (Fig. 9, *SF*) pierces the cone, and the chitin between the pore aperture and the cone, and it ends in the bottom of the pore aperture or pit (Figs. 9–12, *P*) with its peripheral end exposed to the air in the pit.

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[1] All figures, except Text-figs. 1, 2, and 3 are numbered consecutively on Plates I. and II.

The second type of pores is found in the legs of *Orthosoma* (Figs. 13–15), although the pores in the elytra (Fig. 21) and wings (Fig. 31) of the same beetle belong to the first or most common type. The chitinous integument of the legs of *Orthosoma* is thicker than that of the legs of any other beetle examined. Instead of the sense cells (Fig. 13, *SC*) lying in the lumen of the legs outside the pore cavities, in this type they lie inside the pore cavities. When the chitin forming the wall of the pore is not thick enough to protect the entire sense cell, the wall of the pore projects flange-like (Fig. 14, *Fl*) into the lumen of the leg. In Fig. 14 only about one third of the sense cell (*SC*) is shown. Studies of the olfactory pores in various hymenopterous insects made by the writer have shown that the sense cells begin to differentiate at the time when the chitin is beginning to be formed. From this fact, it is quite probable that the sense cells found in the second type of pores have not migrated into the pore cavities, but they now remain in approximately the same position as when the chitin was being formed.

The third type of pores is found in the legs of the lady beetle, *Epilachna borealis*. Instead of the chitin over the external end of the pore being depressed to form a pit, it is elevated dome-like above the surface of the leg. In the center of the dome lies the pore aperture (Fig. 16, *PorAp*). All the pores in the trochanters and most of those in the tibiæ (Fig. 17, *PorAp*) are of this type. Sometimes in the tibia is found a pore whose aperture is on a level with the surface of the tibia. The apertures of all the pores in the elytra (Fig. 18, *PorAp*) and wings (Fig. 19) of this beetle are on a level with the surfaces of the appendages.

As already stated, the olfactory pores of beetles are more or less flasklike as a rule, but there are many variations among them. They may be inverted flask-shaped as found in the legs of *Epilachna* (Figs. 16 and 17) and in the wings of *Passalus* (Fig. 20). Some have the shape of a flask without the neck (Figs. 9, 10 and 12). Some are long and slender like fingers or test tubes (Figs. 11, 18, 19 and 21).

Their sizes also vary much. The length of a pore always depends on the thickness of the chitin. The diameters of the pores of a small beetle (Fig. 25) may be as large, or even larger (Figs. 9 and 10) than the diameters of the pores of a large beetle (Figs. 13–15).

A chitinous cone is always present, although it may sometimes be almost indiscernible. It invariably has the same shade of coloration (Fig. 17, *Con*) as the remaining chitin (Fig. 17, Ch_2) which is formed after the insect has emerged into the imago stage. This is the first time that the writer has been able to determine definitely the formation of the cones. In all the hymenopterous insects studied by the writer, the chitinous integument is practically developed when the insects emerge, but in most beetles only about one third of the chitin is formed when the insects emerge. Since this is true the hypodermal cells are still large and they are rapidly secreting a substance which forms new chitin. Their external ends stand in contact with the chitin, and when no chitin is present they send processes into all holes or cavities in the chitin. Thus the hypodermal cell (Fig. 23, *HypC*) at the mouth of each olfactory pore sends a process into the pore. Since the sense fiber has entered the pore aperture before the cone is formed, the latter is formed at the external end of the pore around the sense fiber. When the chitinous integument (Fig. 17) is fully developed no hypodermal processes run into the pores and the hypodermal cells are very small.

The sense cells are always spindle-shaped (Figs. 12, 13, 16–19 and 23, *SC*). Only occasionally is an entire sense cell seen in a cross section, because the entire cell seldom lies in the same plane as that of the section. More entire sense cells may be seen in longitudinal sections, but even in these the cells are usually cut in two. Entire sense cells were best seen in the oblique sections through the peduncles of the elytra of *Passalus* and *Epilachna*. The nucleus (Figs. 13 and 23, *SCNuc*) of the sense cell is always conspicuous. It may be darker (Fig. 13, *SCNuc*) or lighter (Fig. 23, *SCNuc*) in color than the cytoplasm in the cell. The nucleoli (Fig. 23, *SCNuc*) are also conspicuous.

Smaller sense cells may be seen in the sections through the proximal ends of the trochanters and through the proximal ends of the tibiæ. These (Fig. 17, SC_1) belong to tactile hairs (Fig. 17, *THr*).

In the sections through the legs and elytra of *Epilachna*, gland cells (Fig. 17, *GlC*) are plainly seen in the hypodermis (*Hyp*). These are equally as large as the olfactory sense cells, but they are quite different in structure. The diameters of the pores of the glands (*PorGl*) are slightly smaller than those of the hairs (*PorHr*), and they are much smaller than those of the olfactory pores (*Por*). The morphology and physiology of these gland cells will be given in another paper.

The shapes of the external ends or tops of the pits depend on the shapes of the pore apertures when seen in superficial views. That is, they are round, oblong, slitlike or clublike. The internal ends or bottoms of the pits are always round. The pore aperture, proper, is the round opening leading from the bottom of the pit to the external end of the pore. This aperture is closed by the peripheral end of the sense fiber. The shapes of the pits in cross sections, therefore, depend on the directions in which the microtome knife passes through the pits. The most common shape of a pit in cross section is that of an urn (Fig. 9, *P*). Pits including the pore apertures may be likened to round funnels, or to funnels slightly flattened, or to funnels considerably flattened, or to funnels so flattened that their tops would be club-shaped. In spiders the pits are slits which pass entirely through the cuticula. The sense fibers enter the pore apertures at the bottoms of the slits. The pits or slits in spiders, therefore, may be likened to funnels considerably flattened. When just emerged into the imago stage the pits (Fig. 9, *P*) in the legs generally extend about one-third the distance through the chitin, but when the chitin is fully developed, the pits extend perhaps from one fifth to one eighth the distance through the chitin. In all the figures showing two shades in the chitin, the darker one (Fig. 17, Ch_1) represents the chitin formed at the time when the insect emerges from the last pupal stage, and the lighter one (*Ch_2*) represents the chitin formed after emerging into the imago stage.

As already stated, instead of the olfactory pores of the lady beetle, *Epilachna*, having pits, the chitin over each pore in the legs is elevated domelike above the surface of the leg. The olfactory pores (Figs. 24 and 25) in the legs of the two blister beetles, *Epicauta marginata* and *Epicauta pennsylvanica*, have only indications of pits. Their pore apertures are therefore on a level with the surface of the legs. The olfactory pores in the legs of the potato beetle, *Leptinotarsa 10-lineata*, have shallow pits (Fig. 26, *P*). All four just enumerated species have hypodermal gland pores distributed over the entire body except the wings. These pores are perhaps most abundant on the elytra, but they were never seen on the peduncles of these appendages, and it is quite probable that the secretion from their glands never covers the olfactory pores found on the wings and on the peduncles of the elytra. Judging from the gland pores, the hypodermal glands in the legs of *Epilachna* are more highly developed than are those of the other three species. The gland pores (Figs. 1, 2 and 28, *PorWGl*) on the legs of *Epilachna* lie on all sides and even among the olfactory pores, but in the legs of the other three species the gland pores never lie near the olfactory pores. When examined under a low-power lens the legs and elytra of *Epilachna* appear wet, and many small yellow flakes may be seen on them. The wet appearance is certainly due to the secretion from the hypodermal glands and the flakes are the remains of the secretion after it becomes dry. Thus in *Epilachna* there seems to be a direct correlation between the olfactory pores and the gland pores. Since the pore apertures in the legs lie above the surface of these appendages, the secretion from the hypodermal glands runs away from the pore apertures instead of into them. Such a device enables both sets of organs to function normally without the one hindering the other.

In the legs the sense cells always lie in a blood sinus (Figs. 16 and 17, *BlSin*) some distance from the muscles (Fig. 28, *M*). The nerves (*N*) are easily seen and branches (*NB*) are given off which run to the sense cells (*SC*). The neurilemma (Fig. 17, *Neu*) of the nerve is usually distinct. In the cross section of a nerve, the nervous substance appears more or less netlike and nuclei, probably neuroglia nuclei (Fig. 17, *NeurNuc*), stand out conspicuously in the network. The trachea (Figs. 16, 17 and 28, *Tr*) and nerves (*N* and *NB*) are firmly suspended by the connective tissue whose nuclei (*ConTNuc*) are seen only occasionally. The lumen of the leg at the proximal end of the tibia of *Epilachna* seems to be divided into two chambers by a membrane (Fig. 17, Hyp_1) which resembles hypodermis. This structure has never been seen before by the writer and nothing can be said about its function.

The hypodermis (Fig. 18, *Hyp*) beneath the olfactory pores in the peduncles of the elytra is much thicker than elsewhere. It usually contains all the sense cells (*SC*), but in the elytra of *Passalus* the hypodermis is thinner and since the sense cells are so large and so numerous there is not enough room for all of them in the hypodermis. For this reason only a few of them lie among the hypodermal cells and the remainder of them lie in the lumen of the peduncle between the hypodermis and nerve. As usual they are surrounded by blood. In only one instance was the writer able to trace a sense cell all the way from the pore aperture to the nerve. Fig. 23 represents this sense cell connecting with the pore aperture (*PorAp*) and with the nerve (*N*). The trachea (*Tr*) lies by the side of the nerve. A large nerve (Fig. 29, *N*) and a large trachea (*Tr*) run through the radial plate (*RP*) of the peduncles beneath the olfactory pores. From the nerve many branches are given off which connect with the sense cells.

The hypodermis (Fig. 22, *Hyp*) beneath the olfactory pores in the wings is usually much thicker than elsewhere, but it does not contain the sense cells (*SC*). These cells lie in a blood sinus (Fig. 22, *BlSin*) between the hypodermis (*Hyp*) and the trachea (*Tr*), nerve (*N*) and nerve branches (*NB*). In the wings it is usually difficult to trace a sense fiber all the way to the pore aperture, but in oblique superficial sections this is easily done (Fig. 30). A large nerve and a large trachea run into each wing. These divide so that a smaller nerve and a smaller trachea run through each main vein. The largest trachea (Fig. 31, *Tr*) runs through the subcosta (*Sc*) while the largest nerves (*N*) pass through the veins bearing the olfactory pores. The nerve and trachea run directly beneath the sense cells (*SC*) and from the nerve pass off many branches which connect with the sense cells. In the costa (*C*) and subcosta (*Sc*) where there are no sense cells, only a few nerve fibers can be seen.

In the preceding pages it has been shown that there are many variations in the structure of the olfactory pores of beetles, and that these organs are very similar to those of hymenopterous insects. On the basis of the location of the pore apertures in the integument, the olfactory organs in beetles are intermediate between those of spiders and those of Hymenoptera.

EXPERIMENTS TO DETERMINE THE LOCATION OF THE OLFACTORY ORGANS.

Since it is now generally believed that the olfactory organs of beetles are borne by their antennæ, these appendages of many individuals were pulled off. From one to seven days later, the mutilated insects were tested with odors. In the preceding pages it has been shown that the olfactory pores of Coleoptera are located on the peduncles of the elytra, on the wings and on the legs. In order to ascertain if these structures receive odor stimuli, the elytra, wings and legs were mutilated. One or more days later these mutilated beetles were tested with odors. In all the experiments with unmutilated and mutilated beetles, 434 individuals have been tested. These belonged to 11 species representing eight families.

In order that the behavior of the mutilated beetles would be correctly interpreted, the behavior of unmutilated beetles under experimental conditions was first studied. Since it was not desired to ascertain the relative sensitiveness of males and females, both sexes were used indiscriminately. To determine the relative sensitiveness of unmutilated and mutilated individuals under conditions which permitted of their close observation, triangular experimental cases were employed. These were made of three narrow wooden strips, two of which were five and the third four inches long, each strip being half an inch thick. Wire screen served as a bottom and glass as a top for the case.

The apices and bases of these cases rested on two supports above a rigid table near a window. No screen was used to prevent the beetles from seeing the observer because they never showed any responses to the movements made by the observer.

The following sources of odors were used for determining the reactions of the beetles in the experimental cases; chemically pure essential oils of peppermint, thyme, and wintergreen; parts of plants—leaves and stems of pennyroyal (*Hedoma pulegioides*?), and of spearmint (*Mentha spicata*); decayed matter —parts of decayed beetles (*Harpalus pennsylvanica*). All these substances were kept in stoppered vials of the same shape and size. The leaves and stems of the pennyroyal were dried, but they still gave off a strong odor when the vial was uncorked. The leaves and stems of the spearmint were fresh and they did not emit as strong an odor as did the other substances used. Beetles were killed and were torn to pieces. The pieces were put into a vial and after two or three days they emitted a foul and sickly odor.

A beetle was carefully placed into one of the experimental cases. When first put into the case the insect usually wandered about for several minutes, but finally it became quiet. The insect was tested with the above odors only when it had become perfectly quiet, without the antennæ being moved in the least. The stopper of a vial was quickly removed and the vial was gently and slowly placed under the experimental case directly beneath and within one half inch of the individual being tested. When all of these precautions are taken, a normal beetle generally responds to anyone of these odors within 60 seconds, but when all the reaction times are counted, it is seen that several of them failed to respond within 60 seconds. If a beetle when tested fails to react to an odor within 60 seconds, the response may be regarded as negative, and when it reacts to an odor within 60 seconds, the response may be called positive. As a control, an empty and odorless vial was now and then placed under the insects in the same manner. If by chance a beetle moved while the control test was being made, its behavior was different from that observed when odors were used. Only the first responses have been recorded and in all cases where there was the least doubt as to whether the insect moved for any reason other than the olfactory stimulus, such movements were never recorded. The reaction time was counted in seconds. With an ordinary watch the minimum time which can be definitely recorded is two seconds, although many of the individuals responded to some of the odors much more promptly. Owing to this source of error, the average recorded time is probably double what it should be in the cases where all the responses for the same insect were prompt. An intermission of 10 minutes elapsed between any two tests in the same experimental case. Each individual was tested only once with the same odor.

In recording the responses the term "vibrated" is used to describe the rapid movement of the antennæ or legs up and down or from side to side. When this movement is slow, these appendages are described simply as having "moved." When the antennæ, legs or mouth parts are moved so that they are quickly bent at their articulations, they may be described as being "worked." When at rest a beetle usually lies flat on its thorax and abdomen, so the word "arose" means that the insect gets up and stands on its feet. In the averages of reaction times the probable error is presumably high. It has not been calculated since slight differences in reaction times are not considered as significant in the discussion of results. All anthropomorphic terms are put in quotation marks.

CARABIDÆ.

The Olfactory Sense of *Harpalus pennsylvanica*.

Many ground beetles (*Harpalus pennsylvanica*) were caught under flat stones in a corn field near the laboratory. As soon as brought to the laboratory, 25 of them were placed singly into the experimental cases. As they were being placed into the cases, some of them discharged a substance, presumably from the anal glands, which gave off an odor similar to that from formic acid. Confined in these cases, they sought the dark corners of the cases and did not wander about much inside the cases unless irritated. When half hidden in the dark corners, they rarely responded to odors, so it was necessary to keep them out of the corners while they were being tseted. The longer they remained in the light and the more they were handled, the more satisfactory they were to experiment with. Owing to this kind of behavior, this species and several others used responded more slowly to odors a short time after being caught than they did a few days after being kept in confinement. This fact will explain why some unmutilated beetles just caught respond to odors more slowly than they do two or three days later after having had their antennæ pulled off. The following are the responses of this ground beetle to the odors from the six different substances and the average reaction times in seconds.

Oil of peppermint:
5 moved away quickly.
5 vibrated antennæ.
4 arose quickly.
4 moved slightly.
2 moved antennæ and legs.
1 worked legs.
1 kicked quickly.
1 vibrated antennæ and legs.
1 vibrated legs.
1 jumped slightly.

Reaction time 2 to 10 seconds, average 3.6 seconds.

Oil of thyme:
6 moved away quickly.
5 moved quickly.
5 moved slightly.
2 worked antennæ.
2 moved antennæ and legs.
1 arose slowly.
1 vibrated antennæ.
1 moved backward slowly.
1 worked legs.
1 did not respond.

Reaction time 2 to 60 seconds, average 8.5 seconds.

Oil of wintergreen:
5 moved away quickly.
5 moved slightly.
4 moved away slowly.
3 moved quickly.
2 vibrated legs.
1 arose slowly.
1 stroked antennæ.
1 vibrated antennæ and legs.
1 worked antennæ.
1 worked legs.
1 did not respond.

Reaction time 2 to 60 seconds, average 16.4 seconds.

Leaves and stems of pennyroyal:
10 moved away quickly.
6 moved slightly.
3 vibrated antennæ.
3 worked antennæ.
1 moved away slowly.
1 vibrated legs.
1 did not respond.

Reaction time 3 to 60 seconds, average 21.1 seconds.

Leaves and stems of spearmint:
5 moved away slowly.
5 moved slightly.
3 moved antennæ and legs.
2 moved away quickly.
2 did not respond.
1 worked mouth parts.
1 vibrated legs.
1 vibrated antennæ and legs.

2 worked antennæ.
2 jumped slightly.
1 moved antennæ.

Reaction time 3 to 60 seconds, average 21.8 seconds.

Parts of decayed beetles:
7 moved slightly.
5 did not respond.
4 moved away quickly.
3 moved away slowly.
2 jumped slightly.
1 moved legs.
1 worked antennæ.
1 vibrated legs.
1 vibrated antennæ and worked mouth parts.

Reaction time 5 to 60 seconds, average 28.1 seconds.

The general average reaction time of the 25 beetles tested to the six odors is 16.5 seconds. As a possible reason why one fifth of the individuals tested failed to respond to the odor from the decayed beetles is that these insects probably do not respond to decayed matter unless they are hungry. The 25 beetles tested were put into a wooden box four inches wide, seven inches long and two inches deep. One half inch of moist earth was also put into the box. The beetles soon buried in the earth and from that time on they appeared quite "at home." The box was put into a table drawer where it was more or less dark. About twice each week water was poured upon the earth and the beetles were fed earthworms and various insect larvæ. They drank some of the water and always greedily ate the food given to them. Up to the time of this writing (Jan. 15), 24 of these beetles have died. These lived from 18 to 180 days with 61 days as an average. All the beetles confined in the laboratory have not been fed since Oct. 15, but they have been given water once or twice a week. A few of the dead beetles when removed from the box had been partially eaten, but these insects were never seen fighting one another. While collecting this species in the corn field, a dead one was now and then found.

(*a*) *Effects with Antennæ Pulled Off.*

The antennæ of 25 *Harpalus pennsylvanica* were pulled off at their bases. These insects were then put into a wooden box similar to the one containing the unmutilated individuals just described. This box, also containing moist earth, was placed into the table drawer. The beetles appeared normal in all respects for they drank and ate as greedily as the unmutilated ones and buried in the earth as usual. Seven days later they were placed singly into the experimental cases and were tested with the six odors as usual. They wandered about in the cases slightly more than did the unmutilated ones, but when tested they gave similar responses and reacted just as promptly.

Their reaction times are as follows: Oil of peppermint, 2 to 15 seconds, average 3.8 seconds; oil of thyme, 2 to 25 seconds, average 4.7 seconds; oil of wintergreen, 2 to 25 seconds, average 6.9 seconds; leaves and stems of pennyroyal, 3 to 50 seconds, average 14.4 seconds; leaves and stems of spearmint, 3 to 60 seconds, average 34.9 seconds. Ten failed to respond to this odor. Parts of decayed beetles, 3 to 60 seconds, average 32 seconds. Eight failed to respond to this odor. The general average reaction time of the 25 beetles tested to the six odors is 16.1 seconds. Up to the time of this writing (Jan. 15), 23 of these beetles have died. They lived from 19 to 171 days with 58 days as an average.

(*b*) *Effects with Elytra and Wings Pulled Off.*

The elytra and wings of 25 *Harpalus pennsylvanica*, just collected from the cornfield, were pulled off at their articulations. These mutilated insects were then put into a third box, similar to the two already described. The box was kept in the table drawer with the others. On the following day after mutilating the beetles, they were placed singly into the experimental cases and were tested with the six odors as usual. They seemed normal in all respects except they were extremely restless. Their responses to odors were similar to those of unmutilated ones, except they were slower.

Their reaction times are as follows: Oil of peppermint, 3 to 45 seconds, average 10.7 seconds; oil of thyme, 5 to 50 seconds, average 10.2 seconds; oil of wintergreen, 5 to 60 seconds, average 18 seconds. Two failed to respond to this odor. Leaves and stems of pennyroyal, 5 to 60 seconds, average 29.2 seconds. Seven failed to respond to this odor. Leaves and stems of spearmint, 5 to 60 seconds, average 24.7 seconds. Four failed to respond to this odor. Parts of decayed beetles, 5 to 30 seconds, average 13.4 seconds. The general average reaction time of the

25 beetles tested to all six odors is 17.7 seconds. These mutilated insects lived from 2 to 21 days with 9 days as an average. All the time they were confined in the small box, they drank, ate, and buried in the earth normally, but many times one was seen biting the soft dorsal portion of the abdomen of another. With the elytra and wings removed, the abdomens were unprotected and many of them shrank considerably in size before the beetles died. Some of these beetles were certainly killed on account of the dorsal sides of their abdomens being bitten, because nearly every one found dead had been entirely eaten except the chitinous parts. In the other two boxes as already mentioned, only occasionally was a dead beetle found that had been eaten.

(c) *Effects with Elytra and Wings Pulled Off and Pores on Legs Covered with Vaseline.*

The elytra and wings of 18 *Harpalus pennsylvanica* were pulled off at their articulations. Four days later the trochanters, femurs and proximal ends of the tibiæ of these mutilated beetles were covered with a vaseline-beeswax mixture, consisting of three fourths yellow commercial vaseline and one fourth beeswax. An hour after the legs had been vaselined, the beetles were placed singly into the experimental cases and were tested with the six odors as usual. Most of them were comparatively quiet, but a few were extremely restless. Their responses to odors were not pronounced and were slow, otherwise they were similar to those of unmutilated beetles.

Their reaction times are as follows: Oil of peppermint, 3 to 60 seconds, average 19.5 seconds. Three failed to respond to this odor. Oil of thyme, 3 to 60 seconds, average 12.5 seconds. Two failed to respond to this odor. Oil of wintergreen, 3 to 60 seconds, average 18.7 seconds. Four failed to respond to this odor. Leaves and stems of pennyroyal, 5 to 60 seconds, average 38.6 seconds. Nine failed to respond to this odor. Leaves and stems of spearmint, 3 to 60 seconds, average 32.9 seconds. Seven failed to respond to this odor. Parts of decayed beetles, 4 to 60 seconds, average 22.1 seconds. Two failed to respond to this odor. The general average reaction time of the 18 beetles tested to all six odors is 24.1 seconds. Confined in a box similar to the other three already mentioned, these mutilated beetles drank, ate and buried in the earth normally, but they were less active than unmutilated ones. It was common to see them biting the dorsal sides of the abdomens. Before they died several of their abdomens had shrunk considerably in size. When found dead several of them had been entirely eaten except the chitinous parts. Counting from the time the elytra and wings were pulled off, they lived from 5 to 21 days with 10 days as an average.

The Olfactory Sense of *Harpalus caliginosus*.

Eight ground beetles (*Harpalus caliginosus*) were caught under flat stones. They were tested with the odors from only the three essential oils. In behavior, they were comparatively quiet. When tested, many of them moved away quickly; a few vibrated the antennæ, and a few moved their legs.

Their reaction times are as follows: Oil of peppermint, 2 to 10 seconds, average 4.4 seconds; oil of thyme, 2 to 8 seconds, average 4.1 seconds; oil of wintergreen, 2 to 8 seconds, average 4.1 seconds. The general average reaction time to all three odors is 4.2 seconds. The antennæ of these beetles were pulled off and the insects were then kept in a small box containing earth in the table drawer.

(*a*) *Effects with Antennæ Pulled Off.*

Eight days after the antennæ of the eight preceding *Harpalus caliginosus* had been pulled off, the remaining six live ones were again tested with the same odors in the usual way. Their responses were similar to those given before they were mutilated, but were not so pronounced. When tested with the oil of thyme, one beetle rubbed a hind leg on an elytron for a half minute.

Their reaction times are as follows: Oil of peppermint, 3 to 25 seconds, average 12.5 seconds; oil of thyme, 4 to 60 seconds, average 14.3 seconds. One failed to respond to this odor. Oil of wintergreen, 10 to 35 seconds, average 22.5 seconds. The general average reaction time to all three odors is 16.4 seconds. These mutilated beetles were quite inactive and sometimes scarcely moved when touched with a pencil. They did not eat as greedily as before being mutilated. They lived from 2 to 65 days with 18 days as an average.

COCCINELLIDÆ.

THE OLFACTORY SENSE OF *Epilachna borealis*.

Many lady beetles (*Epilachna borealis*) were caught on pumpkin vines in the corn field. When brought to the laboratory, they were put into a large glass jar near a window. The jar was 11 inches tall and 9 inches in diameter. It was covered with cheesecloth. Since this lady beetle feeds upon the leaves of pumpkin and of allied plants, several pumpkin leaves were put into a wide-mouthed bottle containing water. The bottle with contents was then put into the jar. The beetles soon found the leaves and from that time on, they appeared "at home" as much as they do in corn fields on pumpkin leaves. They were regularly provided with a fresh supply of food. Occasionally they were seen copulating.

On the following day after being caught, 18 of them were removed from the jar and were put singly into the experimental cases. When mechanically irritated they draw in the antennæ and legs, usually eject a small drop of yellowish liquid from each femoro-tibial articulation, and feign death. They may lie apparently lifeless for several moments and when tested with odors they may or may not respond. Owing to this peculiar behavior, they were unsatisfactory to experiment with and their average reaction times are slower than might be expected. They were extremely quiet and when tested they generally moved away slowly. They often vibrated the antennæ and mouth parts, and sometimes the legs.

Their reaction times to the odors from the three essential oils are as follows: Oil of peppermint, 2 to 55 seconds, average 12.4 seconds; oil of thyme, 2 to 20 seconds, average 6.8 seconds; oil of wintergreen, 3 to 60 seconds, average 22.2 seconds. Three failed to respond to this odor. The general average reaction time to all three odors is 13.8 seconds. Sixteen of these insects were mutilated for other experiments. The seventeenth lived only 3 days and the eighteenth is still living at this writing (Jan. 15).

(*a*) *Effects with Antennæ Pulled Off.*

The antennæ of 25 *Epilachna borealis*, just caught, were pulled off at their bases. A small drop of yellowish blood exuded from each wound. On the following day the beetles were tested with odors. As a rule they were so inactive that they appeared lifeless. If touched while moving they feigned death and remained inactive for several moments. When tested with odors most of them worked the mouth parts; some moved away slowly; a few vibrated one or more legs, and some failed to respond.

Their reaction times to the odors from the three essential oils are as follows: Oil of peppermint, 2 to 60 seconds, average 18.6 seconds. Three failed to respond to this odor. Oil of thyme, 2 to 60 seconds, average 38.7 seconds. Fourteen failed to respond to this odor. Oil of wintergreen, 3 to 60 seconds, average 35.1 seconds. The general average reaction time to all three odors is 30.8 seconds. Up to the time of this writing (Jan. 15), 15 of these mutilated beetles have died. They lived from 1 to 96 days with 22 days as an average.

(*b*) *Effects with Elytra and Wings Pulled Off.*

The elytra and wings of 10 *Epilachna borealis* were pulled off at their articulations. A small drop of yellowish blood exuded from each wound. A liquid of the same color is also present throughout the elytra and in the veins of the wings. On the second day after being mutilated, the four remaining live beetles were tested as usual. They were very quiet, but appeared normal in all respects except they responded to odors more slowly than unmutilated ones.

Their reaction times to the odors from the three essential oils are as follows: Oil of peppermint, 10 to 60 seconds, average 25 seconds. One failed to respond to this odor. Oil of thyme, 5 to 60 seconds, average 33.5 seconds. Two failed to respond to this odor. Oil of wintergreen, 7 to 60 seconds, average 35.5 seconds. Two failed to respond to this odor. The general average reaction time to all three odors is 31.3 seconds. Up to the time of this writing (Jan. 15), 1 of these beetles has died. Counting the 7 mutilated beetles that died, they lived from 2 to 3 days with 2 days as an average.

TELEPHORIDÆ.

The Olfactory Sense of *Chaulcognathus pennsylvanica*.

Many fireflies (*Chaulcognathus pennsylvanica*) were caught on goldenrod (*Solidago*). They were put into a cage 20 inches long, 16 inches tall and 12 inches wide. The sides and top of the cage were cheesecloth while the ends and bottom were wood. The cage was kept in the light near a window and a fresh supply of goldenrod was constantly kept in the cage. On the goldenrod in the cage, these insects appeared quite "at home." Twenty-five of them were tested with the odors from the three essential oils. When tested most of them moved away quickly; a few vibrated antennæ; a few vibrated legs, and a few arose slowly. They were extremely restless at all times. In the cage they copulated as freely as they do out-of-doors.

Their reaction times are as follows: Oil of peppermint, 2 to 12 seconds, average 2.6 seconds; oil of thyme, 2 to 10 seconds, average 3 seconds; oil of wintergreen, 2 to 10 seconds, average 3 seconds. The general average reaction time to all three odors is 2.8 seconds. They lived from 3 to 7 days with 3.2 days as an average.

(*a*) *Effects with Antennæ Pulled Off.*

The antennæ of 27 *Chaulcognathus pennsylvanica* were pulled off at their bases. A day later only three were alive. When tested these three responded as promptly as unmutilated ones. The general average reaction time to the odors from the three essential oils is 2.8 seconds. Counting all 27 beetles, they lived from 1 to 5 days with 1.3 days as an average.

LUCANIDÆ.

The Olfactory Sense of *Passalus cornutus*.

Four stag beetles (*Passalus cornutus*) were removed from rotten stumps. While being tested with odors they were comparatively quiet and responded promptly. Their most common response was to draw in the antennæ and to move away slowly. The general average reaction time to all six odors is 3.2 seconds. The antennæ were pulled off at their bases. A small drop of blood exuded from each wound. The beetles were kept in a small box filled with moist rotten wood.

(*a*) *Effects with Antennæ Pulled Off.*

Two days after pulling off the antennæ, the four preceding mutilated beetles were again tested with the same odors. They were more quiet than before being mutilated. Their responses were just as prompt but were less pronounced than before they were mutilated. Their most common response was to work the mouth parts and to move away slowly. The general average reaction time to all six odors is 3.3 seconds. They lived from 4 to 20 days with 12.5 days as an average.

SCARABÆIDÆ.

THE OLFACTORY SENSE OF *Cotinis nitida.*

One lamellicorn beetle (*Cotinis nitida*) was tested with the six odors. The most common response was to stretch out its head, and to move its antennæ and front legs. Once it drew in the antennæ and moved the front legs. The average reaction time is 8 seconds. The antennæ were pulled off at their bases. A small drop of blood exuded from each wound.

(*a*) *Effects with Antennæ Pulled Off.*

A day after pulling off the antennæ, the preceding *Cotinis nitida* was again tested with the same odors. It responded as promptly as before being mutilated. The most common response was to work the mouth parts and to move away slowly. The average reaction time is 8.3 seconds. It lived 12 days after being mutilated.

THE OLFACTORY SENSE OF *Euphoria sepulchralis.*

Five lamellicorn beetles (*Euphoria sepulchralis*) were caught on goldenrod (*Solidago*). While being tested with the odors from the three essential oils, they were extremely restless. They generally moved away slowly and drew in the antennæ when tested with an odor. The general average reaction time is 3.6 seconds. After the antennæ had been pulled off at their bases, the beetles were put into the cage described on page 436.

(*a*) *Effects with Antennæ Pulled Off.*

A day later the five preceding mutilated insects were again tested with the same odors. They were quiet and their responses were similar to those before being mutilated, except, of course, there were no antennal movements. The general average reaction time is 4.3 seconds. These beetles lived from 9 to 42 days with 20 days as an average after being mutilated.

CERAMBYCIDÆ.

The Olfactory Sense of *Cyllene robiniæ*.

Eighteen wood-boring beetles (*Cyllene robiniæ*) were caught on goldenrod (*Solidago*). While being tested with the odors from the three essential oils, they were extremely restless. When tested, most of them moved away quickly; a few arose quickly, and a few vibrated the antennæ. The general average reaction time is 5.4 seconds. These beetles were confined in the cage described on page 436. They were regularly given a fresh supply of goldenrod. They seemed "at home" and copulated as freely in the cage as they do out-of-doors. They lived from 1 to 17 days with 10.4 days as an average.

(*a*) *Effects with Antennæ Pulled Off.*

Eighteen more *Cyllene robiniæ* were collected from goldenrod. Their antennæ were pulled off at the bases. A small drop of blood exuded from each wound. These beetles were placed into the cage with the unmutilated ones. Two days later the 15 remaining live ones were tested with the odors from the essential oils. They were very quiet and their responses were similar to those of unmutilated individuals, except as a rule they were more prompt. The general average reaction time is 3 seconds. In the cage it was common to see the unmutilated and antennæless cerambycids copulating. The former were very active and flew out of the cage whenever the door was opened, but the latter seldom flew and they were not so active. The mutilated ones lived from 1 to 11 days with 5 days as an average.

(*b*) *Effects with Elytra and Wings Pulled Off.*

Eighteen more *Cyllene robiniæ* were collected. Their elytra and wings were pulled off at the articulations. A small drop of blood always exuded from each wound caused by the elytron being pulled off, but only occasionally was blood seen where a wing had been pulled off. A day later when tested with the odors from the three essential oils, these beetles were comparatively quiet and they appeared normal in all respects except in their slowness in responding to odors.

Their reaction times are as follows: Oil of peppermint, 2 to 30 seconds, average 7.1 seconds; oil of thyme, 3 to 20 seconds, average 8.9 seconds; oil of wintergreen 3 to 55 seconds, average 13.4 seconds. The general average reaction time to all three odors is 9.8 seconds. In the cage with the other beetles, these mutilated ones were as active as the unmutilated cerambycids and they were often seen copulating with each other, and with the unmutilated and antennæless ones. They lived from 1 to 11 days with 4.2 days as an average.

CHRYSOMELIDÆ.

The Olfactory Sense of *Leptinotarsa 10-lineata.*

Forty-five Colorado potato beetles (*Leptinotarsa 10-lineata*) were collected in a potato patch near the laboratory. While 25 of them were being tested with the six odors, they were comparatively quiet as a rule, but five were so restless that they were discarded and others were used. Their responses were similar to those of *Harpalus pennsylvanica*, described on page 429.

Their reaction times are as follows: Oil of peppermint, 2 to 7 seconds, average 3.3 seconds; oil of thyme, 2 to 5 seconds, average 3.1 seconds; oil of wintergreen, 2 to 12 seconds, average 5 seconds; leaves and stems of pennyroyal, 4 to 60 seconds, average 26.7 seconds. Six failed to respond to this odor. Leaves and stems of spearmint, 2 to 60 seconds, average 25.6 seconds. Seven failed to respond to this odor. Parts of decayed beetles, 5 to 60 seconds, average 27.9 seconds. Seven failed to respond to this odor. The general average reaction time of the 25 beetles tested to all six odors is 15.4 seconds. These insects were confined in a cage in the light near a window. This cage is 30 inches long, 30 inches high and 4½ inches wide. All six sides are wire-screen. A fresh supply of potato plant leaves was constantly kept in the cage. The beetles confined in this cage on the potato plant leaves appeared "at home" just as much as they do in potato patches. They ate the leaves, copulated and laid eggs as usual. Up to the time of this writing (Jan. 15), 28 of the 45 beetles have died. These lived from 14 to 151 days with 69 days as an average.

(*a*) *Effects with Antennæ Pulled Off.*

Twenty-nine more potato beetles were collected from the potato patch. Their antennæ were pulled off at the bases. A small drop of blood exuded from each wound. These insects were put into the wire-screen cage with the unmutilated ones. Two days later the 23 remaining live ones were tested with only the odors from the three essential oils. All of these beetles were quite inactive and three failed to respond when tested. These three also failed to respond when touched with a pencil. For this reason they were discarded. The general average reaction time of the 20 beetles tested is 3.5 seconds. As a rule these mutilated insects appeared normal in all respects several days after having the antennæ pulled off, because they ate, copulated and were as active as ever. They lived from 2 to 140 days with 38 days as an average.

(*b*) *Effects with Elytra Pulled Off and Wings Cut Off.*

Thirty-one more potato beetles were collected. Their elytra were pulled off at the articulations and the wings were cut off as closely as possible to the articulations. A small drop of reddish or yellowish blood exuded from each wound. The heavy veins, extending from the base of the wing to where the wing folds, contain most of the blood found in these wings. The elytra are also filled with blood. The amount of blood in them gradually diminishes from the base to the distal end. A day after being mutilated 25 of these insects were tested with the six odors. They were apparently normal in all respects except in their slowness in responding to odors. They were as active as unmutilated ones and eight were extremely restless. Their responses were similar to those of unmutilated beetles, except they were not pronounced.

Their reaction times are as follows: Oil of peppermint, 2 to 40 seconds, average 7.8 seconds; oil of thyme, 2 to 15 seconds, average 4.8 seconds; oil of wintergreen, 3 to 60 seconds, average 21.1 seconds. Five failed to respond to this odor. Leaves and stems of pennyroyal, 5 to 60 seconds, average 32.2 seconds. Ten failed to respond to this odor. Leaves and stems of spearmint, 3 to 60 seconds, average 29.8 seconds. Eight failed to respond to this odor. Parts of decayed beetles, 3 to 60 seconds, average 30.4 seconds. Eight failed to respond to this odor. The general average reaction time of the 25 beetles tested to the six odors is 22.7 seconds. In the wire-screen cage with the other potato beetles already tested, these mutilated ones appeared normal, because they ate normally and copulated as much as usual. Since the soft dorsal sides of their abdomens were unprotected, many of them soon began to sink, so that by the time a beetle died, the abdomen had shrunk to about one-fourth its original size. Up to the time of this writing (Jan. 15), 29 of these 31 mutilated insects have died. They lived from 3 to 140 days with 52 days as an average.

(*c*) *Effects with Elytra Pulled Off, Bases of Wings Glued and Pores on Legs Covered with Vaseline.*

Twenty-nine more potato beetles were collected. Their elytra were pulled off at the articulations. Two days later the upper surfaces of the bases of the wings of the 26 remaining live ones were covered with liquid glue. Since the olfactory pores extend a considerable distance from the base of the wing along the radial vein, the glue applied probably did not cover more than 90 per cent. of the pores on each wing. Three hours after applying the glue, the trochanters, femurs and proximal ends of the tibiæ of these beetles were covered with the vaseline-beeswax mixture. An hour still later the insects were tested with the six odors. They were as active as unmutilated ones and appeared normal in all respects except in their responses to odors. Their responses were never pronounced and seldom prompt.

Their reaction times are as follows: Oil of peppermint, 3 to 60 seconds, average 10.7 seconds. One failed to respond to this odor. Oil of thyme, 3 to 60 seconds, average 9 seconds. One failed to respond to this odor. Oil of wintergreen, 5 to 60 seconds, average 35.9 seconds. Eleven did not respond to this odor. Leaves and stems of pennyroyal, 3 to 60 seconds, average 35.2 seconds. Twelve did not respond to this odor. Leaves and stems of spearmint, 5 to 60 seconds, average 42.6 seconds. Fourteen failed to respond to this odor. Parts of decayed beetles, 5 to 60 seconds, average 40.3 seconds. Fourteen failed to respond to this odor. The general average reaction time of the 26 beetles tested to the six odors is 29 seconds which is twice the reaction time of unmutilated potato beetles to the same odors. When the reaction times to the odors from only the three essential oils are considered, these mutilated insects responded only one fifth as rapidly as did the unmutilated ones. In the wire-screen cage with the other potato beetles already tested, they were apparently normal as long as they lived, because they ate and copulated as usual and were always as active as the unmutilated ones. Before they died their abdomens shrunk considerably in size. Up to the time of this writing (Jan. 15), 28 of the 29 have died. These lived from 2 to 151 days with 61 days as an average.

MELOIDÆ.

The Olfactory Sense of *Epicauta marginata*.

Twenty blister beetles (*Epicauta marginata*), commonly known as the "old-fashioned potato bugs," were caught on clematis. When mechanically irritated, they fold the antennæ and legs against the body, usually eject a small drop of amber-colored liquid from each femoro-tibial articulation, and feign death. On account of this behavior, they were unsatisfactory to experiment with. When put into the experimental cases, some of them lay apparently lifeless for almost a half day. In this state they never respond to any odor, and after becoming as active as usual, they may or may not respond to odors.

When tested with the odors from only the three essential oils, a general average reaction time of 13.9 seconds was obtained. Two of them failed to respond to each of the oils of peppermint and wintergreen. These insects were confined in the cage described on page 436. They were regularly provided with a fresh supply of clematis. In this cage on the clematis they seemed "at home," but they flew out at every opportunity.

They copulated as usual. They lived from 11 to 40 days with 27.6 days as an average.

(*a*) *Effects with Antennæ Cut Off.*

Eight more *Epicauta marginata* were collected. Their antennæ were cut off at the bases. A small drop of amber-colored blood exuded from each wound. Seven days later the two remaining live ones were tested with the odors from the three essential oils. The general average reaction time is 5 seconds. All these beetles were abnormal in behavior. They lived from 1 to 8 days with 3.4 days as an average.

(*b*) *Effects with Antennæ Pulled Off.*

The antennæ of 12 more *Epicauta marginata* were pulled off at their bases. A small drop of blood exuded from each wound. When tested with the odors from the essential oils three days later, the eight remaining live beetles gave a general reaction time of 5.9 seconds. They were less abnormal in behavior than those with the antennæ cut off. They lived from 2 to 13 days with 5.5 days as an average.

(*c*) *Effects with Elytra and Wings Pulled Off.*

The elytra and wings of nine *Epicauta marginata* were pulled off at their articulations. A small drop of blood exuded from each wound. When tested with the odors from the essential oils two days later, the seven remaining live beetles gave a general reaction time of 25.7 seconds. Two of them failed to respond to each of the oils of peppermint and wintergreen. These mutilated insects appeared normal in behavior and in confinement they copulated as usual. They lived from 2 to 14 days with 8 days as an average.

THE OLFACTORY SENSE OF *Epicauta pennsylvanica*.

Twenty-five blister beetles (*Epicauta pennsylvanica*) were caught on golden rod (*Solidago*). This species has the same habit of feigning death when mechanically irritated as has *Epicauta marginata*. When tested with the odors from the essential oils, they gave a general average reaction time of 11.5 seconds which is only one-half as rapid as the reaction time of the same species devoid of antennæ. Three failed to respond to the oil of peppermint, one to the oil of thyme and two to the oil of wintergreen. A common response was to vibrate the legs. They were placed into the cage with the other species of blister beetles. They were regularly provided with a fresh supply of goldenrod. In the cage they appeared normal, and they copulated as much as usual. They lived from 2 to 25 days with 11.2 days as an average.

(*a*) *Effects with Antennæ Pulled Off.*

The antennæ of 30 *Epicauta pennsylvanica* were pulled off at their bases. When tested with the odors from the essential oils three days later, the 22 remaining live beetles gave a general reaction time of 5.3 seconds. They were only slightly abnormal in behavior. They lived from 2 to 25 days with 8.7 days as an average.

(*b*) *Effects with Elytra and Wings Pulled Off.*

The elytra and wings of 21 *Epicauta pennsylvanica* were pulled off at their articulations. A small drop of blood exuded from each wound. Blood was also seen in the distal ends of the elytra. When tested with the odors from the essential oils two days later, the 17 remaining live beetles gave a general reaction time of nine seconds. One of them failed to respond to the oils of thyme and wintergreen. These insects appeared normal in confinement with the other blister beetles. They copulated as usual. They lived from 1 to 33 days with 10.7 days as an average.

A summary of all the preceding experiments to determine the location of the olfactory organs in beetles is best presented in a tabulated form. The following table is such a summary. Since a comparison of the behavior of unmutilated and mutilated insects alone is not always a safe criterion for judging the general behavior of mutilated beetles, the behavoir of the mutilated beetles recorded in this table is based mostly upon a comparison of the longevities of unmutilated and mutilated individuals of the same species. A "+" after a figure in the last column means that all the insects used in the experiment have not yet died. The longevity is based only on those that have died up to the time of this writing (Jan. 15).

TABLE II.

SUMMARY OF EXPERIMENTS TO DETERMINE THE LOCATION OF THE OLFACTORY ORGANS IN COLEOPTERA.

Species.	Experiment and Behavior of Insects Tested.	Average Reaction Time.		No. of Individuals Tested.	Average Length of Life in Captivity.
		For Three Odors.	For Six Odors.		
		Sec.	Sec.		Days.
Harpalus pennsylvanica.	Unmutilated. Normal in behavior	9.5	16.5	25	61.0+
	Antennæ pulled off. Normal in behavior	5.1	16.1	25	58.0+
	Elytra and wings pulled off. Slightly abnormal in behavior	13.0	17.7	25	9.0
	Elytra and wings pulled off and pores on legs covered with vaseline. Slightly abnormal in behavior	16.9	24.1	18	10.0
Harpalus caliginosus.	Unmutilated. Normal in behavior	4.2		8	Used below
	Antennæ pulled off. Slightly abnormal in behavior	16.4		6	18.0
Epilachna borealis.	Unmutilated. Normal in behavior	13.8		18	Used below
	Antennæ pulled off. Slightly abnormal in behavior	30.8		25	22.0+
	Elytra and wings pulled off. Slightly abnormal in behavior	31.3		4	2.0+
Chaulcognathus pennsylvanica.	Unmutilated. Normal in behavior	2.8		25	3.2
	Antennæ pulled off. Slightly abnormal in behavior	2.8		3	1.3
Passalus cornutus.	Unmutilated. Normal in behavior	3.0	3.2	4	Used below
	Antennæ pulled off. Slightly abnormal in behavior	3.0	3.3	4	12.5
Cotinis nitida	Unmutilated. Normal in behavior	5.0	8.0	1	Used below
	Antennæ pulled off. Normal in behavior	5.6	8.3	1	12.0
Euphoria sepulchralis.	Unmutilated. Normal in behavior	3.6		5	Used below
	Antennæ pulled off. Normal in behavior	4.3		5	20.0
Cyllene robiniæ	Unmutilated. Normal in behavior	5.4		18	10.4
	Antennæ pulled off. Slightly abnormal in behavior	3.0		15	5.0
	Elytra and wings pulled off. Slightly abnormal in behavior	9.8		18	4.2

Species.	Experiment and Behavior of Insects Tested.	Average Reaction Time. For Three Odors. Sec.	Average Reaction Time. For Six Odors. Sec.	No. of Individuals Tested.	Average Length of Life in Captivity. Days.
Leptinotarsa 10-lineata.	Unmutilated. Normal in behavior	3.8	15.4	25	69.0+
	Antennæ pulled off. Normal in behavior	3.5		20	38.0
	Elytra pulled off and wings cut off. Normal in behavior	11.2	22.7	25	52.0+
	Elytra pulled off, bases of wings glued and pores on legs covered with vaseline. Normal in behavior	18.5	29.0	26	61.0+
Epicauta marginata.	Unmutilated. Normal in behavior	13.9		20	27.6
	Antennæ cut off. Considerably abnormal in behavior	5.0		2	3.4
	Antennæ pulled off. Slightly abnormal in behavior	5.9		8	5.5
	Elytra and wings pulled off. Slightly abnormal in behavior	25.7		7	8.0
Epicauta pennsylvanica.	Unmutilated. Normal in behavior	11.5		25	11.2
	Antennæ pulled off. Slightly abnormal in behavior	5.3		22	8.7
	Elytra and wings pulled off. Normal in behavior	9.0		17	10.7

A summary of the preceding table shows the following: After the antennæ were pulled off, four of the 11 species tested were normal and seven were slightly abnormal in behavior. After the elytra and wings were pulled off one species was normal while four were slightly abnormal in behavior. After the elytra were pulled off and the wings were cut off, the one species tested was normal in behavior. After the elytra and wings were pulled off and the pores on the legs were covered with vaseline, the one species tested was slightly abnormal in behavior. After the elytra were pulled off, the bases of the wings glued and the pores on the legs covered with vaseline, the one species tested was normal in behavior.

Four unmutilated species responded to odors more slowly than did the same species after the antennæ had been pulled off. This is explained by the fact that most beetles are more or less "timid" for some time after being caught, and some feign death. As a rule the longer they are confined and the more they are handled, the more satisfactory they are to experiment with. Five species without antennæ responded to odors as promptly as did the same species unmutilated. Two species without antennæ responded to odors more slowly than did the same species unmutilated. Since these were abnormal in behavior and judging from the reaction times of the other nine species with antennæ pulled off, it is only reasonably to attribute the slow reaction times of these two species to their abnormal condition caused by the antennæ being pulled off. The six species so mutilated that most of their olfactory pores on the elytra and wings were prevented from functioning responded from two to five times more slowly than did the same species unmutilated or with the antennæ pulled off. The two species so mutilated that most of their olfactory pores on the elytra, wings and legs were prevented from functioning responded from two to six times more slowly than did the same species unmutilated or with the antennæ pulled off.

From all the preceding results, it seems that the antennæ do not carry any of the olfactory organs, while the olfactory pores found on the peduncles of the elytra, on the dorsal surfaces of the wings, on the trochanters, tibiæ, sometimes on the femurs and tarsi, and perhaps on the mouth appendages, are the true olfactory organs in beetles.

SUMMARY.

In making a comparative study of the olfactory pores in beetles, 50 species belonging to 47 genera and representing 34 families were used. A group of pores is always present on the peduncle of each elytron. It lies on the dorsal side of the well-exposed radial plate. The number of pores on a pair of elytra varies from 12 to 310. As a rule, the more pores in the group the smaller they are and the closer they are together.

Of the 47 winged species examined, 11 have only one group of pores on each wing, 21 have two groups on each wing, 12 have three groups on each wing, and 3 have four groups on each wing. These groups are always located on the dorsal surface. Only occasionally are a few scattered pores found on the ventral side of a wing. When one or two groups are present, they lie on the radius. When three groups are present, all three may lie on the radius, or two may lie on the radius and the third on the media. When four groups are present, one lies on the subcosta, two on the radius and one on the media. The largest group on the radius usually extends nearly all the way to the fold of the wing and sometimes all the distance to the fold. The number of pores on a pair of wings varies from 130 to 982.

There are usually two groups of pores at the proximal end of each trochanter. Sometimes a pore is found at the proximal end of the femur. It is common to find a few pores at the proximal end of each tibia; and sometimes pores are found in the tibial spines and on the tarsi. The number of pores on all six legs varies from 49 to 341.

In regard to water beetles, the better the legs are adapted for locomotion in water, the fewer pores they have. The smallest winged species (*Coxelus*) examined has 273 pores, which is the smallest number of all the species, and the largest species (*Orthosoma*) has 1,268 pores which is the largest number of all the species examined. The apterous species have more pores on the legs than usual. As a rule, the smaller the species, the fewer its pores and the larger they are, comparatively speaking. As a rule, there are no generic and specific differences, except variations in number of pores, the amount of variation depending on the sizes of the individuals compared. There are no individual and sexual differences other than slight variations in number of pores.

The pore apertures or pits are round, oblong, slitlike or club-shaped. On the elytra and wings they are always round or oblong. On the legs they have all four of the enumerated shapes.

The spindle-shaped sense cells of most beetles lie in the lumens of the appendages outside the pore cavities, but in the legs of *Orthosoma* the sense cells lie inside the pore cavities. A small chitinous cone is always present. It is formed by the hypodermal cell at the mouth of the pore after the insect has emerged from the last pupal stage, and at the same time when the chitinous integument is being considerably thickened. The sense cells are fully developed when the insect emerges into the imago stage. The sense fiber pierces the cone and the layer of chitin between the pore aperture and cone, and it enters the bottom of the pore aperture or pit where its peripheral end comes into direct contact with the outside air. In Hymenoptera the sense fibers enter the pore apertures which are almost on a level with the external surface of the chitin. In Coleoptera, with a few exceptions, the sense fibers enter the bottoms of pits which lie in the chitin one third (at time of emerging into imago stage) the distance from the external surface. In the legs of the lady beetle, *Epilachna borealis*, instead of the chitin which surrounds the pore apertures being depressed, it is elevated so that the pore apertures lie in the center of domes above the general surface of the legs. In the legs of the blister beetles, *Epicauta marginata* and *E. pennsylvanica*, the pore apertures lie on a level with the surface of the legs. In the legs of the potato beetle, the pore apertures lie at the bottoms of shallow pits. All four preceding species have hypodermal gland pores over the entire body, except the wings. These pores in the lady beetle are perhaps the most highly developed. They lie on all sides and even among the olfactory pores on the legs. In the other three species they are less highly developed on the legs near the olfactory pores and none is found very close to an olfactory pore. This correlation between the hypodermal gland pores and the olfactory pores is certainly a means of preventing the secretion from the gland cells from running into the pore apertures.

A large nerve and a large trachea run into each elytron and wing. In the peduncle of the elytron they run through the radial plate just beneath the group of olfactory pores. Branches from the nerve are given off which connect with the sense cells. The large nerve and trachea passing into the wing soon divide so that a smaller nerve and a smaller trachea run through each main nerve. The largest trachea passes through the subcosta, and the largest nerves pass through the veins carrying the olfactory pores. These nerves give off branches which connect with the sense cells. The sense cells wherever found are always surrounded by blood.

In the experiments to determine the location of the olfactory organs, 434 individuals were tested. These belonged to 11 species representing 8 families. After the antennæ were pulled off, 4 of the 11 species tested were normal and 7 were slightly abnormal in behavior. After the elytra and wings were pulled off 1 species was normal while 4 were slightly abnormal in behavior. After the elytra were pulled off and the wings were cut off, the 1 species tested was normal in behavior. After the elytra and wings were pulled off and the pores on the legs were covered with vaseline, the 1 species tested was slightly abnormal in behavior. After the elytra were pulled off, the bases of the wings glued and the pores on the legs covered with vaseline, the 1 species tested was normal in behavior.

Four unmutilated species responded to odors more slowly than did the same species after the antennæ had been pulled off. This is explained by the fact that most beetles are more or less "timid" for some time after being caught, and some feign death. As a rule, the longer they are confined and the more they are handled, the more satisfactory they are to experiment with. Five species without antennæ responded to odors as promptly as did the same species unmutilated. Two species without antennæ responded to odors more slowly than did the same species unmutilated. Since these were abnormal in behavior and judging from the reaction times of the other 9 species with antennæ pulled off, it is only reasonable to attribute the slow reaction times of these two species to their abnormal condition caused by the antennæ being pulled off. The 6 species so mutilated that most of their olfactory pores on the elytra and wings were prevented from functioning responded from 2 to 5 times more slowly than did the same species unmutilated or with the antennæ pulled off. The two species so mutilated that most of their olfactory pores on the elytra, wings and legs were prevented from functioning responded from 2 to 6 times more slowly than did the same species unmutilated or with the antennæ pulled off.

From all the preceding results, it seems that the antennæ do not carry any of the olfactory organs, while the olfactory pores found on the peduncles of the elytra, on the dorsal surfaces of the wings, on the trochanters, tibiæ, sometimes on the femurs and tarsi, and perhaps on the mouth appendages, are the true olfactory organs in beetles.

DISCUSSION.

Since the writer ('14*c*) has already written a complete review of all the literature available concerning the sense of smell in insects, only a brief discussion is necessary in this paper.

Hicks ('57) says that the olfactory pores in Coleoptera are arranged in long rows along the subcostal nerves. The same author ('59) states that in Coleoptera these organs are highly developed and occur in numerous groups on the subcostal vein, mostly at the widest part, but are also scattered along it to the fold of the wing. In *Carabus* they are found on veins other than the subcostal. In many beetles the pore is overarched by a hair, which probably protects the organ. He could distinguish no sexual differences in these organs, except the pores are slightly larger in the females, due to their greater size. Hicks ('60) first found the olfactory pores on the legs of beetles. The present writer has never seen a hair overarching an olfactory pore.

Hochreuther ('12) seems to be the first to study the internal anatomy of the olfactory pores in beetles. Since he used only *Dytiscus marginalis* and perhaps because he did not have enough sections through these organs, he failed to understand their anatomy. He states that each dome-shaped organ is located at the bottom of a chitinous flask, the mouth of which communicates with the exterior. Instead of the peripheral end of the sense fiber coming into direct contact with the air in the flask, it apparently stops just beneath the chitinous dome at the top of the organ. His terminal strand (Terminalstrand) may be the same as the hypodermal secretion forming the cone described by the writer. Hochreuther found a few of these dome-shaped organs on the epicranium near the margin of the eyes, nine on the proximal end of the first antennal segment, two on the distal end of the second antennal segment, a few on the dorsal side of the labrum, a very few on the dorsal side of the mandible, several on each maxilla, about 18 on the first four segments of the front legs, about 10 on the first three segments of the middle legs, and a few on the trochanters of the hind legs. He evidently did not examine the wings. Thus according to Hochreuther these organs are rather widely distributed. Since the peripheral ends of the sense fibers do not come into contact with the outside air, but connect with the tops of the domes, he suggests that they receive some kind of mechanical stimuli, although he performed no experiments to determine their function.

Lehr ('14), resuming the search for sense organs in *Dytiscus marginalis* where left off by Hochreuther, found dome-shaped organs on the elytra and wings. He found three main groups in identically the same places as described by the present writer. The number of pores in the group on the elytron varies from 130 to 150. The two main groups on the radius (his subcosta) of the wing are large, but he did not count the pores in them. He found a fourth group, consisting of about 30 pores, on the ventral side of the costa near the base of the wing. He also found a few scattered pores on the dorsal side of the costa just distal to the fold of the wing, a few on the second cubitus, and a few irregularly scattered along the full length of the media. Lehr has described the anatomy of these organs almost identically as seen by the present writer, but it seems that he has not correctly interpreted some of the structures. He seems to think that each sense cell is surrounded by another cell, but the latter cell is perhaps nothing more than coagulated blood and the portion of it extending into the pore is certainly a hypodermal secretion forming the cone as described in the preceding pages. His neurilemma nuclei are perhaps hypodermal nuclei. He is able to trace the sense fiber through the cone, but he has not recognized the small opening through the dome. This is not surprising, because the pores in the wings as so small that the openings or pore apertures are never noticed unless first seen in the largest pores in the legs or mouth parts. In the thinnest sections, the chitin forming the dome is so thick as compared to the diameter of the pore aperture that the aperture appears only as a streak slightly lighter than the other chitin in the dome. Lehr has nothing to say about the physiology of these organs.

In experimenting with mutilated beetles, Hauser ('80) seems to be the only one who has taken their longevity into consideration. And even he has not kept an accurate record of their behavior and longevity. He claims to have studied the behavior of beetles before and after the removal of the antennæ. When the antennæ were removed he ascertained that many beetles soon became sick and died, while others lived thereafter for many days. When tested with odors, most of the beetles without antennæ failed to respond, but Hauser states that *Carabus*, *Melolontha* and *Silpha* still responded to odors, although more slowly.

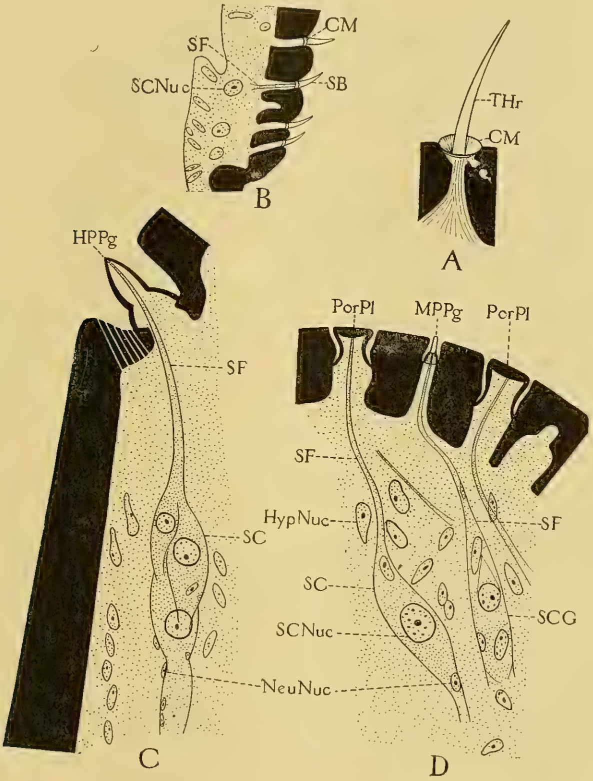

Fig. 3. Antennal organs of the water beetle, *Dytiscus marginalis*, copied from Hochreuther (1912). *A*, small tactile hair (Sinneshaar) from first segment of antenna, total preparation (Fig. 1 from Hochreuther), × 330; *B*, portion of Fig. 12 from Hochreuther, showing four small sense bristles (Sinnesborsten) from proximal end of second segment of antenna, × 265; *C*, longitudinal section (Fig. 48 from Hochreuther) through a hollow pit peg (hohlen Grubenkegel), × 470; *D*, longitudinal section through a small massive pit peg (massiven, grubenständigen Zapfen) and two pore plates (kelchförmige Organe), × 590. This drawing is a combination of Figs. 32 and 58 from Hochreuther. Only the pore plates (*PorPl*) are taken from Fig. 58. Hochreuther gives a drawing of only one perfect pore-plate organ, or cup-shaped organ, and it is from the maxillary palpus. *CM*, cup-shaped membrane; *HPPg*, hollow pit peg; *MPPg*, massive pit peg; *PorPl*, pore plate; *SB*, sense bristle; *THr*, tactile hair. See page 456 for other abbreviations.

For the purpose of judging whether the antennal organs are better adapted anatomically than the olfactory pores for receiving odor stimuli, the former organs (Text-fig. 3, p. 453), of *Dytiscus marginalis* have been copied from Hochreuther ('12). This work of Hochreuther is a comprehensive study of the morphology of all the chitinous sense organs of *Dytiscus*. Since it is perhaps the latest and certainly the best study on the antennal organs of beetles, these organs shall be briefly described.

Each of the 11 segments in the antenna of *Dytiscus* carries a number of sense organs. The farther from the base of the antenna the more numerous they are. The distal half of the antenna is covered abundantly with sense organs, while the proximal half is sparingly covered with them. The first and second segments are well provided with slender tactile hairs (Text-fig. 3*A*, *THr*) which have been called *Sensilla trichodea* by Schenk. These hairs are also found on all the other appendages and even on the head, thorax and abdomen. Two groups of sense bristles (Text-fig. 3*B*, *SB*), called *Sensilla chœtica* by Schenk, lie at the proximal end of the second segment. These hairs are also common on most of the other appendages, on the head, thorax and abdomen. All segments, except the first one, are well provided with small massive pit pegs of the thick-walled type (Text-fig. 3*D*, *MPPg*). All segments, except the first one, are only sparingly provided with a second type of pit pegs. This one is the hollow or thin-walled type (Text-fig. 3*C*, *HPPg*). Only about six of these were found on each segment. Besides being found on the antennæ, both types of pit pegs are common on all the mouth parts, on the mesothorax, around the spiracles, on all the legs, and on the sexual apparatus. Pit pegs have been called *Sensilla coelloconica* by Schenk. All segments, except the first two, are abundantly supplied with the cup-shaped or pore-plate organs (Text-fig. 3*D*, *PorPl*). For both antennæ they are estimated between 4,500 and 5,000. These organs are also common on the palpus of the first maxilla. They were first studied by Nagel on the antennæ and maxillary palpi of Dytiscidæ. In the honey bee Schenk has called them *Sensilla placodea*. Of the five antennal organs of *Dytiscus*, only the hollow pit pegs are regarded by Hochreuther as probably olfactory in function. If they really act as olfactory organs, then the mouth parts, thorax, legs and sexual organs must aid in receiving odor stimuli. Hochreuther considers the antennæ more important as appendages for carrying organs for receiving mechanical stimuli rather than those receiving chemical stimuli.

According to various authors the antennal organs of different beetles vary only slightly. The antennal organs of *Dytiscus* are also similar to those of the honey bee. In both of these insects the tactile hairs are of the same type. The Forel flasks and pit pegs of the honey bee are two types of pit pegs which are perhaps rudimentary, because the tips of the hairs do not come to the exterior of the chitin. The massive pit pegs, hollow pit pegs, and the sense bristles of *Dytiscus* are certainly nothing more than three types of tactile hairs. The hollow pit pegs compare closely with the pegs of the honey bee, except the pegs have thinner chitin at the tips. This is probably on account of more acute sense of touch in the honey bee. The pore-plate organs of the honey bee and the cup-shaped organs of *Dytiscus* are also quite similar.

One or more of the antennal organs of every insect studied have been called olfactory organs, and it is possible that most of these organs may be found on other appendages, besides the antennæ, as already seen in *Dytiscus*.

In conclusion it seems beyond a doubt that none of the antennal organs of beetles shown in Text-fig. 3 serves as an olfactory organ, and that the olfactory pores are well adapted anatomically for receiving odor stimuli, because the peripheral ends of their sense fibers come into direct contact with the external air.

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EXPLANATION OF PLATES I. AND II.

All figures including Text-figs. 1 and 2 are from camera lucida drawings made at the base of the microscope. Figures 1 to 8 inclusive and 22 on the plates are enlarged 465 diameters. All the remaining figures on the plates, except the diagrams 28, 29 and 31, are enlarged 580 diameters.

ABBREVIATIONS.

BlSin	blood sinus.
BM	basal margin of elytron.
C	costa.
Ch	chitin.
Ch_1	chitin formed before insect emerges into imago stage.
Ch_2	chitin formed after insect emerges from last pupal stage.
ChM	chitinous membrane of pore plate.
CM	cup-shaped membrane of tactile hair on antenna.
Con	chitinous cone.
ConT	connective tissue.
ConTNuc	nucleus of connective tissue.
Cx	coxa.
F	femur.
Fl	flange of olfactory pore.
Fo	where wing folds.
GlC	gland cell.
HPPg	hollow pit peg on antenna.
Hr	hair.
Hyp	hypodermis.
Hyp_1	membrane resembling hypodermis which divides the lumen of proximal end of the tibia of *Epilachna* into two chambers.
HypC	hypodermal cell.
HypNuc	hypodermal nucleus.
HypS	hypodermal secretion.
M	muscle.
MD	muscle disk.
Me	media.
Mo	mouth of pore.

MPPg	small massive pit peg on antenna.
N	nerve.
NB	nerve branch.
Neu	neurilemma.
NeuNuc	nucleus of neurilemma.
NeurNuc	neuroglia nucleus.
P	pit of pore.
PorAp	pore aperture.
PorGl	pore of gland.
PorHr	pore of hair.
PorPl	pore plate on antenna.
Por	pore of olfactory organ.
PorW	pore wall.
PorWGl	pore wall of gland.
PorWHr	pore wall of hair.
R	radius.
RP	radial plate.
SB	small sense bristle on antenna.
SC	sense cell.
SC_1	sense cell of tactile hair
SCG	sense cell group.
SCNuc	sense cell nucleus.
SCNucl	sense cell nucleolus.
Sc	subcosta.
ScH	subcostal head.
SF	sense fiber.
Tar	tarsus.
Tb	tibia.
TbSp	tibial spine.
THr	tactile hair.
Tr	trachea.
TrNuc	Nucleus of trachea.
Tro	trochanter.
1 to 6	groups Nos. 1 to 6 of the olfactory pores.
a	location of scattered pores on ventral side of wing.
b and *c*	location of scattered pores on tibia.

PLATE I.

FIG. 1. Six of the eight olfactory pores (*PorW*) in group 6 on inner surface of right hind leg of *Epilachna borealis;* also one hair (*Hr*), one hair socket (*porWHr*) and two hypodermal gland pores (*PorWGl*).

FIG. 2. Two olfactory pores (*PorAp*), five hairs (*PorWHr*) and 19 gland pores (*PorWGl*) on outer surface at proximal end of right hind leg of *Epilachna*.

FIG. 3. Five olfactory pores from tibial spine of *Epicauta marginata* (same as shown in Text-fig. 2*G*).

FIG. 4. Group 1 of olfactory pores on peduncle of elytron of *Epilachna* (same as shown in Text-fig. 1*B*).

FIG. 5. Seven of the olfactory pores in group 2 on wing of *Epilachna*.

FIG. 6. Eleven of the olfactory pores in group 3 on wing of *Epilachna*.

FIG. 7. Ten of the olfactory pores in group 4 on wing of *Epilachna*.

FIG. 8. Four of the five olfactory pores on ventral side of wing of *Epilachna*. Figs. 5 to 8 represent some of the pores as shown in Text-Fig. 1*C*.

FIG. 9. Olfactory pore from trochanter of *Uloma*.

FIG. 10. Olfactory pore from tibia of *Uloma*.

FIG. 11. Three olfactory pores from elytron of *Uloma*.

FIG. 12. Olfactory pore and sense cell from wing of *Uloma*.

FIG. 13. Olfactory pore and sense cell from trochanter of *Orthosoma* (cut slightly obliquely).

FIG. 14. Olfactory pore and about one third of sense cell (*SC*) from trochanter of *Orthosoma*, showing pit (*P*) and flange (*Fl*).

FIG. 15. Olfactory pore from tibia of *Orthosoma*.

FIG. 16. Oblique section through trochanter of *Epilachna*, showing anatomy of leg. It was cut in such a manner that no muscles are shown in the section and that the nerve (*N*) is severed in two places.

FIG. 17. Cross section through proximal end of tibia of *Epilachna*, showing anatomy of leg at this place. The gland pore (*PorGl*), hair pore (*PorHr*) and sense cells (*SC*), belonging to the tactile hairs (*THr*) were taken from two other sections, and the gland cell just beneath the gland pore was taken from the other end of this section.

FIG. 18. Four olfactory pores and a small portion of hypodermis from elytron of *Epilachna*. The material used for Figs. 17 and 18 was from an old adult beetle that had been confined in the laboratory nearly all summer.

FIG. 19. Four olfactory pores, sense cells and nerve (*N*) from wing of *Epilachna*.

PLATE I.

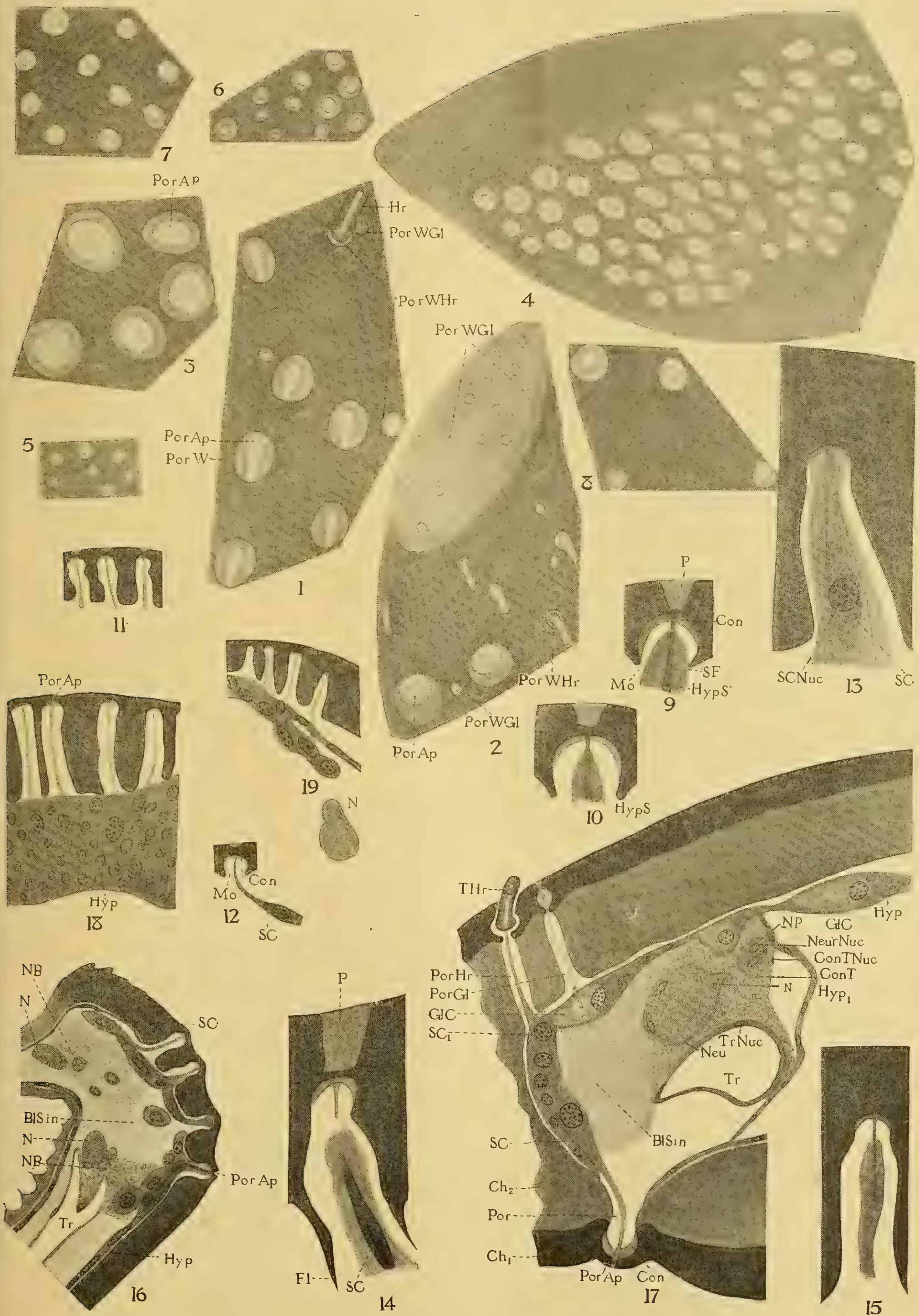
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PLATE II.

FIG. 20. Three olfactory pores from wing of *Passalus*.

FIG. 21. Six olfactory pores from elytron of *Orthosoma*.

FIG. 22. Cross section through wing of *Orthosoma*, showing anatomy of wing beneath olfactory pores.

FIG. 23. Olfactory pore from elytron of *Passalus*, showing sense cell (*SC*) connected with pore aperture (*PorAp*) and with nerve (*N*); also hypodermal cell (*HypC*) that forms the cone (*Con*).

FIG. 24. Olfactory pore from trochanter of *Epicauta marginata*.

FIG. 25. Olfactory pore from trochanter of *Epicauta pennsylvanica*.

FIG. 26. Olfactory pore from trochanter of *Leptinotarsa 10-lineata*.

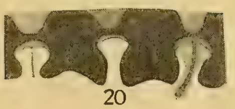
FIG. 27. Three olfactory pores from tibial spine of *Epicauta marginata*. The material used for Figs. 24 to 27 had been treated with caustic potash.

FIG. 28. Transverse-longitudinal diagram of proximal end of trochanter belonging to right hind leg of *Epilachna*, showing internal anatomy of leg and superficial view of hairs, hair sockets, gland pores and olfactory pores. The four pores at the right belong to group 6 and the three at the left belong to group 5.

FIG. 29. Oblique transverse-longitudinal diagram of portion of peduncle belonging to *Epilachna*, showing internal anatomy of radial plate (*RP*), innervation of olfactory pores and a superficial view of a few of the pores in group 1. The transverse portion of the diagram passes through the radial plate in the direction of the line marked "a" in text Fig. 1*B*.

FIG. 30. Oblique superficial view of olfactory pores on wing of *Epilachna*, showing sense fibers (*SF*) connected with pore apertures (*PorAp*).

FIG. 31. Transverse-longitudinal diagram of portion of wing belonging to *Orthosoma*, showing internal anatomy of wing, innervation of olfactory pores and a superficial view of a few of the pores on radius (*R*) and media (*Me*).



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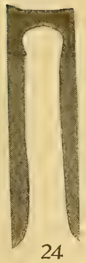

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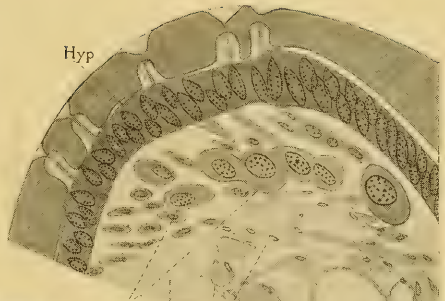

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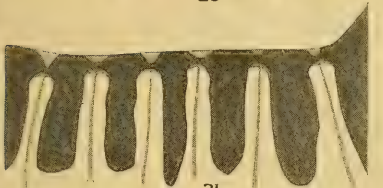

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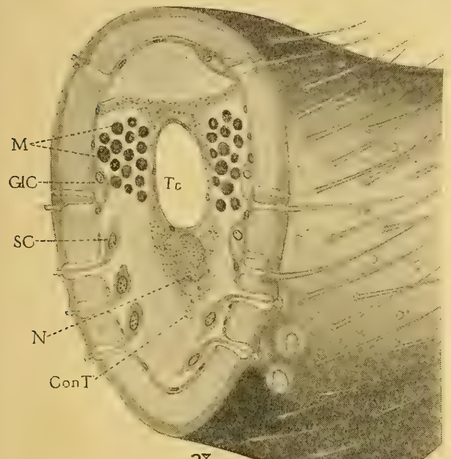

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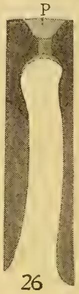

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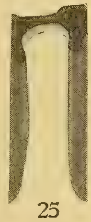

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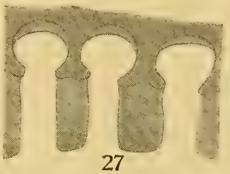

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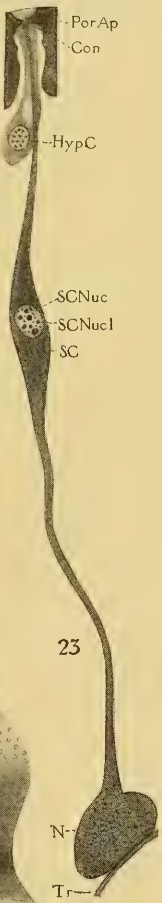

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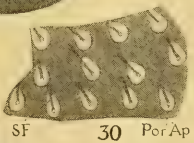

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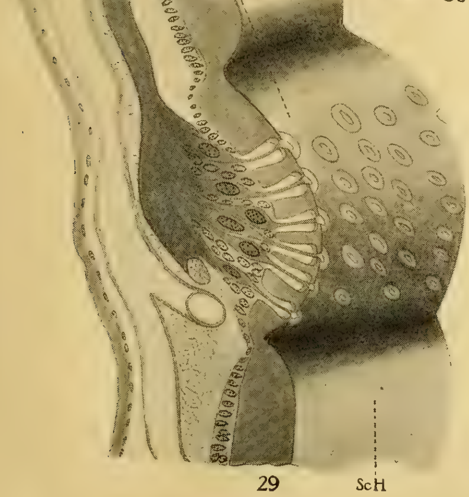

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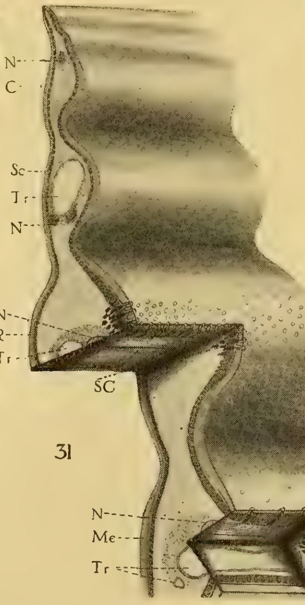

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